Impact of complex yeast nutrient products on selected non-*Saccharomyces* yeasts

by

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Declaration

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Summary

In recent years there has been a growing interest in non-*Saccharomyces* yeasts for winemaking due to their ability to produce more complex wines. These yeasts, considered weak fermenters, are used in combination with *Saccharomyces cerevisiae* and compete for nutrients such as nitrogen. Therefore, it is important for the winemaker to know what nutrients may be insufficient so that corrective action can be taken. Yeast assimilable nitrogen (YAN), a growth limiting resource naturally occurring in grape must, is important for yeast metabolism as well as for production of desirable aromatic compounds. When YAN is deficient it can lead to slow or stuck fermentations and production of undesirable compounds. Thus, to ensure a complete alcoholic fermentation and desirable aroma profile, nitrogen supplementation is required. Traditionally, ammonium salts are added as a nitrogen supplement, however, recently several complex yeast nutrients have also become commercially available. These yeast nutrients are yet to be investigated for fermentation with non-*Saccharomyces* yeasts. This study investigated the impact of eight complex commercial yeast nutrients on three commercial non-*Saccharomyces* yeasts (*Torulaspora delbrueckii* Biodiva™ TD291, *Pichia kluyveri* Viniflora® Frootzen™ and *Metschnikowia pulcherrima* Flavia® MP346). Fermentations were carried out with single yeasts or combined with *S. cerevisiae* in sequential fermentations in synthetic grape must. The *M. pulcherrima* sequential fermentation was repeated in Chenin blanc grape must. For the single yeast fermentations, it appeared that the nutrients had a greater effect on the onset of fermentation than on the growth of the yeasts and that one nutrient (nutrient treatment Y2) was preferred by all the yeasts. This is the first time that nitrogen supplementation at the same level but with different content was investigated for non-*Saccharomyces* wine yeast sequential fermentations. The ability of non-*Saccharomyces* yeasts to persist in sequential fermentations could be improved with nutrient selection. Further investigations with *M. pulcherrima* sequential fermentations in Chenin blanc must found clear differences for the two different matrices. Although synthetic must is a defined medium that reduces the risk of unknown variables, it is not a true representation of how these nutrients can influence non-*Saccharomyces* yeasts in real grape must. Nutrient selection can also increase desirable esters and influence the sensory properties of wine; however, this should be further investigated and confirmed through sensory evaluation. This study improved the current knowledge of non-*Saccharomyces* yeasts and their utilisation of complex yeast nutrients. It demonstrated that nutrient selection can improve non-*Saccharomyces* yeast implantation as well as improve production of desirable volatiles.

Opsomming

In onlangse jare was daar 'n toenemende belangstelling in nie-*Saccharomyces* giste vir wynmaak doeleindes weens hul vermoë om meer komplekse wyn te produseer. Hierdie giste, beskou as swak fermenteerders, word gewoonlik met *Saccharomyces cerevisiae* gebruik en kompeteer vir voedingstowwe soos stikstof. Dit is dus belangrik vir die wynmaker om te weet watter voedingstowwe tekort is, sodat korrektiewe stappe geneem kan word. Gis assimileerbare stikstof is 'n groei beperkende hulpbron wat natuurlik in druiwe mos voorkom en is belangrik vir gis metabolisme sowel as die produksie van verlangde aromatiese verbindings. Wanneer gis assimileerbare stikstof onvoldoende is, kan dit lei tot stadige of swak fermentasies en die produksie van ongewenste verbindings. Dus, om te verseker dat alkoholiese fermentasie eindig met 'n gewenste aromatiese profiel, word stikstof aanvullings vereis. Tradisioneel word ammonium sout gebruik vir stikstof aanvulling, daar is egter onlangs verskeie komplekse gis voedingstowwe ook kommersieel beskikbaar gestel. Hierdie gis voedingstowwe is nog nie ondersoek vir fermentasies met nie-*Saccharomyces* giste nie. Die huidige studie het die invloed van ag komplekse kommersiële gis voedingstowwe op drie nie-*Saccharomyces* giste (*Torulaspora delbrueckii* Biodiva™ TD291, *Pichia kluyveri* Viniflora® Frootzen™ en *Metschnikowia pulcherrima* Flavia® MP346) ondersoek. Fermentasies was uitgevoer of met 'n enkel gis of in kombinasie met *S. cerevisiae* in opeenvolgende fermentasies in sintetiese druiwe mos. Die *M. pulcherrima* opeenvolgende fermentasie was herhaal in Chenin blanc druiwe mos. Vir die enkel gis fermentasies het dit gebleik dat die voedingstowwe 'n groter effek op die aanvang van fermentasie gehad het as op die groei van die giste en dat een van die voedingstowwe (voedingstof behandeling Y2) verkies was deur al die giste. Hierdie is die eerste studie wat stikstof aanvulling op dieselfde vlak, maar met verskillende inhoud ondersoek was vir nie-*Saccharomyces* wyn gis opeenvolgende fermentasies. Die vermoë van die nie-*Saccharomyces* giste om voort te duur in opeenvolgende fermentasies kon verbeter word met selektiewe gebruik van voedingstowwe. Verdere ondersoek met *M. pulcherrima* opeenvolgende fermentasies in Chenin blanc het duidelike verskille getoon tussen die twee verskillende matrikse. Alhoewel sintetiese druiwe mos 'n gedefinieerde medium is wat die risiko van onbekende veranderlikes verminder, bly dit steeds nie 'n ware verteenwoordiger van hoe hierdie voedingstowwe nie-*Saccharomyces* giste kan beïnvloed in regte druiwe mos nie. Selektiewe gebruik van voedingstowwe kan ook verlangde esters vermeerder en die sensoriese eienskappe van die wyn beïnvloed; dit moet egter nog verder ondersoek word en bevestig word

deur sensoriese evaluering. Hierdie studie verbeter die huidige kennis van nie-*Saccharomyces* giste en hoedat hul komplekse voedingstowwe benut. Dit het verder ook gedemonstreer hoe selektiewe gebruik van voedingstowwe die inplantasie van nie-*Saccharomyces* giste kan verbeter sowel as die produksie van verlangde vlugtige verbindings.

This thesis is dedicated to my parents, who always supported and believed in me.

Biographical sketch

Louisa Beukes was born in Nababeep on 08 December 1992. She matriculated from Namaqualand High School in 2011 thereafter she obtained her BSc degree in Molecular Biology and Biotechnology from Stellenbosch University in 2015. She then did a one-year internship at the Agricultural Research Council's institute ARC Infruitec-Nietvoorbij, where her interest in wine microbiology was nurtured. Following this, she completed a BSc (Hons) degree in Wine Biotechnology at Stellenbosch University. Louisa then commenced with an MSc in Wine Biotechnology in 2017 at Stellenbosch University.

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Preface

This thesis is presented as a compilation of 3 chapters.

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Chapter 1

Short literature review with project aim and objectives

Chapter 1. Short literature review and project aim

1.1 Short literature review

Non-*Saccharomyces* yeasts are native yeasts found on grape berries and in grape must and have gained interest for winemaking purposes as they are considered to produce more complex wines (Jolly *et al.* 2006; Ciani *et al.* 2010; Jolly *et al.* 2014). Indeed, mixed fermentations with *Torulaspora delbrueckii* has been shown to improve the complexity, mouthfeel and overall quality of the resulting wine when compared to fermentations with only *Saccharomyces cerevisiae* (Azzolini *et al.* 2015; Belda *et al.* 2015; Arslan *et al.* 2018). Similar results have also been found for *Metschnikowia pulcherimma* mixed fermentations (Benito *et al.* 2015; Ruiz *et al.* 2018). Most non-*Saccharomyces* yeasts cannot complete alcoholic fermentation and it is recommended that *S. cerevisiae* should also be inoculated, either simultaneously or sequentially (Jolly *et al.* 2006). The timing of *S. cerevisiae* inoculation in these mixed fermentations have been found to influence the fermentation capability of both the non-*Saccharomyces* as well as *S. cerevisiae*, suggesting that there could be competition for nutrients such as nitrogen (Medina *et al.* 2012; Lleixà *et al.* 2016).

The nitrogen requirements of some non-*Saccharomyces* yeasts and mixed fermentations have been investigated to some degree (Medina *et al.* 2012; Lleixà *et al.* 2016; Gobert *et al.* 2017; Rollero *et al.* 2018; Prior *et al.* 2019). Rollero *et al.* (2018) and Prior *et al.* (2019) found that non-*Saccharomyces* yeasts that are considered stronger fermenters, have similar nitrogen preference and uptake as *S. cerevisiae*, while weaker fermenters have very low nitrogen uptake. It has also been shown that the nitrogen source preferences of non-*Saccharomyces* yeasts can vary significantly between species (Gobert *et al.* 2017; Rollero *et al.* 2018). However, non-*Saccharomyces* yeasts are not yet as well studied as *S. cerevisiae* and consequently most of the available knowledge is related to *S. cerevisiae*.

It has been widely reported that the minimum nitrogen that *S. cerevisiae* requires to complete fermentation, is 140 mg N/L (Jiranek *et al.* 1995; Bell and Henschke 2005; Tesnière *et al.* 2015). Yeast assimilable nitrogen (YAN), is often one of the growth limiting resources naturally occurring in

grape must which can vary significantly in concentration (Bell and Henschke 2005). The total YAN of grape must consists mainly out of ammonium ions and free alpha amino nitrogen (FAN) from amino acids (Bell and Henschke 2005; Vilanova *et al.* 2007; Tesnière *et al.* 2015). Yeasts can also utilise low molecular weight peptides. When YAN is deficient, it can lead to slow or stuck fermentations and production of undesirable compounds such as hydrogen sulphide (H_2S) (Jiranek *et al.* 1995; Barbosa *et al.* 2012; Sturgeon *et al.* 2013). To avoid this situation, nitrogen is added to fermentations in the form of ammonium salts, usually di-ammonium phosphate (DAP) (Bell and Henschke 2005).

The use of ammonium salts to supplement the YAN before or during fermentation has been extensively investigated for *S. cerevisiae* fermentations (Bely *et al.* 2003; Taillandier *et al.* 2007; Martínez-Moreno *et al.* 2014; Andorrá *et al.* 2018; Seguinot *et al.* 2018). The concentration of ammonium supplementation to the must has been investigated, either using a wide range of concentrations in synthetic must (Vilanova *et al.* 2007) or by increasing the nitrogen to moderate or high levels in grape must (Torrea *et al.* 2011; Vilanova *et al.* 2012). Vilanova *et al.* (2007) and Torrea *et al.* (2011) found that when YAN increases with ammonium supplementation, the fermentation duration decreases, and the yeast consumes all the FAN and ammonium available. However, contrasting results were found in Albariño must, as the ammonium supplementation showed no effect on fermentation duration and had high residual FAN and ammonia (Vilanova *et al.* 2012). This could be due to the complexity and composition differences between synthetic and grape must, as well as the higher initial YAN in the grape must study.

Different ammonium supplementation strategies to the synthetic must, or at the start of stationary phase, have also been investigated (Martínez-Moreno *et al.* 2014; Seguinot *et al.* 2018). The different supplementation strategies can be described as appropriate (increasing YAN from 100 mg N/L to around 200 mg N/L), or over supplementation (increasing YAN from 200 mg N/L to around 500 mg N/L). Over supplementation could occur when grape must YAN is unknown, while appropriate supplementation could prevent stuck fermentation when grape must YAN is too low (Martínez-Moreno *et al.* 2014). Appropriate supplementation later in fermentation can also result in

higher glycerol and reduced acetic acid (Martínez-Moreno *et al.* 2014) or reduced fermentation duration and biomass (Seguinot *et al.* 2018). Although over supplementation greatly reduced the fermentation time, more so for late addition than early addition, it resulted in high acetic acid concentrations with residual ammonium.

Ammonium supplementation at different concentrations also impacts production of volatile compounds. Overall, higher ammonium concentrations, leads to decreased higher alcohols, branched chain acids and their esters (Vilanova *et al.* 2007; Torrea *et al.* 2011; Vilanova *et al.* 2012). Vilanova *et al.* (2007) and Torrea *et al.* (2011) also found that the acetate esters, medium chain fatty acids (MCFA) and their esters were higher for ammonium supplemented fermentations, with the exception of 2-phenylethyl acetate that decreased. Of interest is that the results by Torrea *et al.* (2011), show that the MCFA esters were higher at moderate YAN levels (320 mg N/L) than at high YAN levels (480 mg N/L). Although these esters are present at low concentrations in wines, they can contribute significantly to fruity characteristics due to their low odour threshold (2 – 30 µg/L) (Guth 1997; Ferreira *et al.* 2000). Thus, supplementation with DAP to achieve moderate YAN can improve sensory properties of wine. The timing of DAP addition can also reduce H_2S when added later in fermentation compared to addition to must (Mendes-Ferreira *et al.* 2010).

Although these studies provide a general understanding of how ammonium addition can improve fermentation, they only investigated one or two *S. cerevisiae* strains. The study by Taillandier *et al.* (2007) found strain variation for fermentation kinetics when YAN was increased with ammonium chloride. The different commercial *S. cerevisiae* were either unaffected by YAN concentration, had improved fermentation capabilities with increased YAN, or had an optimal YAN concentration. This is likely a result of the strain variation for nitrogen requirement, as it can be either relatively high or low, or it can be for a specific source (Jiranek *et al.* 1995; Crépin *et al.* 2012; Gutiérrez *et al.* 2012).

There has also been studies that investigated amino acid supplementation on its own (Hernández-Orte *et al.* 2005; Garde-Cerdán and Ancín-Azpilicueta 2008; Seguinot *et al.* 2018) or in combination with ammonium (Beltran *et al.* 2005; Hernández-Orte *et al.* 2005; Rollero *et al.* 2015). When amino acids were supplemented to low YAN grape must, the fermentation kinetics were similar to the

control fermentation and both total esters and fatty acids were directly proportional to the concentration of amino acids added (Garde-Cerdán and Ancín-Azpilicueta 2008). However, when compared to ammonium supplementation, the amino acid supplementation did not improve ester production (Hernández-Orte *et al.* 2005; Miller *et al.* 2007), but had reduced propanol concentrations and similar acetate ester production as ammonium (Seguinot *et al.* 2018). Furthermore, supplementation with amino acids and ammonium, compared to only ammonium, produced lower acetic acid and higher ethyl and acetate esters and higher alcohols (Torrea *et al.* 2011).

Current international wine legislation does not permit the addition of amino acids to wine fermentations. However, the addition of yeast derivatives is allowed and are commercially available as complex yeast nutrients. These complex nutrients consist mainly of either inorganic nitrogen, organic nitrogen or a combination of these and can also contain vitamins. The inorganic nitrogen is generally an ammonium salt such as DAP or ammonium sulphate, while the organic nitrogen is from yeast derivatives (inactivated or autolysed yeasts). Yeast derivatives can however also contain lipids, mannoproteins and peptides (Belviso *et al.* 2005; Del Barrio-Galán *et al.* 2012; Kevvai *et al.* 2016). These commercial products have been developed for *S. cerevisiae* and although they have been widely used in the wine industry, almost no research has been published. There are even less reports on how the nutrients affect non-*Saccharomyces* yeasts in wine fermentation. It is possible that competition for nutrients between non-*Saccharomyces* and *S. cerevisiae* could be partially or completely alleviated with proper nutrient supplementation. The focus of this study was therefore to investigate the impact of different commercial nutrient products on different non-*Saccharomyces* wine yeasts and to better understand how they influence the fermentation.

1.2 Problem statement

To date, most research has focused on the nitrogen requirements of *S. cerevisiae* although those of certain wine-relevant non-*Saccharomyces* wine yeasts have recently been investigated. Competition for nutrients in various inoculation scenarios has also been considered. Traditionally, ammonium is added to a fermentation to help the yeast complete fermentation or produce a better-quality wine. Several complex yeast nutrients are also available. However, very limited published data are

available concerning these complex commercial nutrient products and their impact on non-*Saccharomyces* wine yeast and final product of fermentation. Further research is required to better understand these complex nutrients and how they influence the fermentation capabilities and aroma/flavour production of different commercial non-*Saccharomyces* wine yeasts.

1.3 Aim and objectives

The aim of this study was to investigate the impact of different commercial yeast nutrients on selected non-*Saccharomyces* yeasts and *S. cerevisiae*. Four commercial wine yeasts were screened in synthetic grape must using different inoculation strategies. The four commercial wine yeasts included three non-*Saccharomyces* yeasts and one *S. cerevisiae*. The investigation was then pursued on real grape must and one non-*Saccharomyces* yeast and *S. cerevisiae* combination in order to determine how the results obtained in the synthetic grape juice correlated with real grape juice. To achieve this aim, the study had the following objectives:

Objective 1:

Determining the YAN concentrations and nitrogen composition of eight commercial yeast nutrient products from different manufacturers, commonly used in the South African wine industry.

Objective 2:

Determining the influence of the eight different complex commercial yeast nutrient products and DAP on the fermentation kinetics of selected non-*Saccharomyces* yeasts and *S. cerevisiae* using different inoculation strategies, listed below as A and B, in synthetic must.

A. Individual yeast inoculation strategy
B. Co-inoculation (sequential) strategy with a non-*Saccharomyces* and *S. cerevisiae*

Objective 3:

Determining the influence of eight different complex commercial yeast nutrient products and DAP on the fermentation kinetics of one non-*Saccharomyces* yeast and *S. cerevisiae* combination (as determined in objective 2) and the resulting metabolites in a small laboratory scale fermentation in real grape must.

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Chapter 2

Research results:

Impact of complex yeast nutrient products on selected non-*Saccharomyces* yeasts

Chapter 2. Research results: Impact of complex yeast nutrient products on selected non-*Saccharomyces* yeasts

2.1 Introduction

Over the last few years, the use of non-*Saccharomyces* yeasts for wine making has gained great interest. Indeed, some species have been found to reduce volatile acidity, increase glycerol production and improve aromatic complexity of wines (Sadoudi *et al.* 2012; Benito *et al.* 2015; Varela *et al.* 2017; Esccribano *et al.* 2018). Subsequently several of these yeasts, including *Torulaspora delbrueckii*, *Lachancea thermotolerans* and *Metschnikowia pulcherrima*, have been commercialised. However, most non-*Saccharomyces* yeasts cannot finish alcoholic fermentation and must be inoculated along with *Saccharomyces cerevisiae*, either simultaneously or sequentially. It is thus likely that competition for nutrients occurs between the different yeast species during fermentation.

Nitrogen is one of the growth-limiting nutrients found in grape must and 140 mg N/L is the accepted minimum assimilable nitrogen concentration required by *S. cerevisiae* to finish alcoholic fermentation (Bely *et al.* 1990; Bell and Henschke 2005). However, during sequential fermentations, non-*Saccharomyces* yeasts consume nitrogen before *S. cerevisiae* inoculation. For instance, yeasts such as *T. delbrueckii* and *L. thermotolerans* are considered strong fermenters and can consume the majority of the available nitrogen within the first 48 h following inoculation (Prior *et al.* 2019). In contrast, yeast such as *M. pulcherrima* and *Pichia kluyveri* are considered weaker fermenters and consume a lot less nitrogen (Prior *et al.* 2019). Upon inoculation of *S. cerevisiae*, if the assimilable nitrogen is insufficient, sluggish and even stuck fermentations can be observed (Taillandier *et al.* 2014).

Nitrogen deficiency is generally amended with the addition of ammonium salts. However, it has been shown that supplementation with both amino acids and ammonium salts produce wines with more desired sensory properties compared to only ammonium supplementation (Hernández-Orte *et al.* 2005; Torrea *et al.* 2011). The addition of amino acids to wine fermentations is only allowed in the form of yeast derivatives. Addition of yeast derivatives in the form of inactivated yeast three days

after the start of fermentations were found to improve the fermentation capability of the yeast, with reduced acetic acid concentrations (Belviso *et al.* 2005). Another study found that *S. cerevisiae* can also utilise the peptides found in yeast derivatives as source of nitrogen (Kevvai *et al.* 2016).

Several studies have recently reported on the nitrogen requirements of non-*Saccharomyces* yeasts (Lleixà *et al.* 2016; Gobert *et al.* 2017; Rollero *et al.* 2018; Prior *et al.* 2019). However, only one study has investigated actual yeast derivative addition on fermentation with non-*Saccharomyces* yeast (Zara *et al.* 2014). The authors investigated the effect of diammonium phosphate (DAP) and yeast derivative additions at the start of fermentation on the growth and fermentation performance of *Candida zemplinina* (also known as *Starmerella bacillaris*). The yeast derivative resulted in improved non-*Saccharomyces* viability and higher glycerol concentrations compared to other nutrient treatments (Zara *et al.* 2014).

Most manufacturers of active dried wine yeasts also supply yeast nutrients consisting of ammonium salts and/or yeast derivatives. These complex yeast nutrients have been developed for *S. cerevisiae*. However, with the increasing use of non-*Saccharomyces* yeast for wine fermentation, whether these non-*Saccharomyces* can utilise and benefit from these nutrients should be investigated. This would enable wine producers to make an informed decision on the use of these nutrients. The aim of this study was to investigate the impact of various complex commercial yeast nutrient supplementation products on different non-*Saccharomyces* wine yeast fermentations. The study was carried out on single yeasts as well as on sequential fermentations with *S. cerevisiae* in synthetic grape must. Additional fermentations were completed in Chenin blanc grape must.

2.2 Materials and methods

2.2.1 Commercial yeast nutrients

A total of eight commercial yeast nutrients were investigated. These nutrients were divided into three groups based on their nitrogen content as per the manufacturer's technical information sheet (Table 2.1). The yeast assimilable nitrogen (YAN) concentration of the commercial nutrients was determined by the formol titration method (Schnierda *et al.* 2014). Each analysis was performed in

duplicate and repeated twice with DAP (0.15 g/L) as control. The amino acid concentrations of the nutrients were analysed by high performance liquid chromatography (HPLC) as described in Prior *et al.* (2019). The ammonium concentrations were determined with the enzymatic assay, Enzytec™ Fluid Ammonia (Id-No: E5390, R-Biopharm, Germany) on the Arena 20XT (Thermo Electron Oy, Finland) automated enzymatic kit robot.

Table 2.1. Summary of the content, based on manufacturer's technical information sheet, of commercial yeast nutrients used in the study.

Content	**Yeast derived**			**Yeast derived with mineral salt**				**Mineral salt**
	Y – 1	**Y – 2**	**Y – 3**	**YM – 1**	**YM – 2**	**YM – 3**	**YM – 4**	**M – 1**
Yeast derivative	Yeast autolysate	Yeast autolysate	Yeast hulls	Inactivated yeasts	Inactivated yeasts	Inactivated yeasts	Inactivated yeasts	N/A[1]
Mineral salt	N/A	N/A	N/A	DAP[2]	DAP	DAP	DAP + AS[3]	AS
Vitamin	N/A	N/A	N/A	Thiamine	Thiamine	Thiamine	N/A	Thiamine

[1] Not applicable, [2] diammonium phosphate, [3] ammonium sulphate.

2.2.2 Yeast and preculture conditions

The four commercial wine yeasts (three non-*Saccharomyces* yeasts and one *S. cerevisiae*) used in this study are listed in Table 2.2. The yeasts were maintained on yeast extract peptone dextrose (YPD) agar (Merck, Modderfontein, South Africa) slants at 4°C for the duration of the study. To prepare for inoculation, the yeasts were grown in 10 ml YPD broth (Merck) at 30°C for 24 h before being transferred into 250 ml YPD broth for overnight growth at 22°C with shaking at 125 rpm. The cells were harvested by centrifugation (1381 *g* for 5 min), washed twice with 0.9% NaCl solution (saline) and resuspended in 10 ml saline. The aforementioned saline was filtered through a 0.22 µm cellulose nitrate syringe filter (GVS Filter Technology, Sanford, USA) to improve the flow-cytometry results. The cell count was determined by flow-cytometry using the Muse® Cell Analyzer (Merck) according to the manufacturer's instructions.

Table 2.2. Selected commercial wine yeast of this study.

Yeast species	**Strains**	**Manufacturer**
Saccharomyces cerevisiae	Lalvin® EC1118	Lallemand (Blagnac, France)
Torulaspora delbrueckii	Level 2 Solution Biodiva® TD291	Lallemand
Pichia kluyveri	Viniflora® Frootzen™	Chr. Hansen (Hørsholm, Denmark)
Metschnikowia pulcherrima	Level 2 Solution Flavia® MP346	Lallemand

2.2.3 Fermentation conditions

A synthetic grape must was used for laboratory-scale fermentations and the base must (without nitrogen) was as described by Henschke and Jiranek (1993) (Table 2.3). The nitrogen content was based on Bely *et al.* (1990), with YAN concentration reduced to 140 mg N/L (Table 2.4). Ten nitrogen treatments were used in the study comprising eight commercial yeast nutrients (Table 2.5) and two additional controls. Each nutrient was added within the individual manufacturer's recommended dosage (Table 2.5) to result in a final value of 45 mg N/L. For the Y category nutrients that had low YAN values, the highest manufacturer's recommended dosage was used and supplemented with DAP, so that the YAN was the same for all nutrient treatments. The two controls consisted of DAP addition at 45 mg N/L (positive control, C+) and no further nitrogen addition (negative control, C-).

Each commercial non-*Saccharomyces* wine yeast was inoculated on its own, or sequentially with the *S. cerevisiae*, into the synthetic grape must for each nitrogen treatment. The *S. cerevisiae* was investigated as a control and for the sequential fermentations, inoculated 48 h after the non-*Saccharomyces* yeast. Inoculum for all the yeasts were 1 x 10^6 cells/mL. The fermentation volume was 80mL in a 100mL sterilized glass bottle. The fermentations were carried out in triplicate under self-induced anaerobiosis at 22°C with shaking at 120 rpm. All fermentations were done similarly to ensure results are comparable. An un-inoculated treatment was included in each experiment to monitor weight loss by evaporation. The loss due to evaporation was factored into the weight loss calculations.

The *M. pulcherrima* sequential yeast combination was further investigated in Chenin blanc grape must. The Chenin blanc grape must (220 g/L sugar, 150 mg N/L YAN, 4.54 g/L total acidity and pH 3.36) was obtained from the Nietvoorbij Research Cellar (ARC Infruitec-Nietvoorbij, Stellenbosch). The nitrogen composition is provided in Addendum A, Table A2.2. The grape must was stored at -20°C and thawed at 15°C overnight, homogenised and sterilised by sequentially filtering through 0.45-µm and 0.22-µm cellulose nitrate filters (Sartorius Stedim Biotech, Göttingen, Germany) before use. Fermentation conditions were the same as for the synthetic fermentations.

Table 2.3. Composition of synthetic grape must medium (without nitrogen) based on Henschke and Jiranek (1993).

Carbon (g/L)		**Lipids (mg/L)**	
Glucose	100	β-Sitosterol	10.0
Fructose	100	Tween 80	10.7
Acid (g/L)		**Salts (g/L)**	
KH-Tartrate	2.50	K_2HPO_4	1.14
L-Malic acid	3.00	$MgSO_4 \cdot 7H_2O$	1.23
Citric acid	0.20	$CaCl_2 \cdot 2H_2O$	0.44
Vitamins (mg/L)		**Trace elements (µg/L)**	
Myo-inositol	100.00	Manganese (II) chloride tetrahydrate	200
Pyridoxine hydrochloride	2.00	Zinc chloride	135
Nicotinic acid	2.00	Iron (II) chloride	30
Calcium pantothenate	1.00	Copper (II) chloride	15
Thiamine hydrochloride	0.50	Boric acid	5
PABA.K	0.20	Cobalt (II) nitrate hexahydrate	30
Riboflavin	0.20	Sodium molybdate dehydrate	25
Biotin	0.13	Potassium iodate	10
Folic acid	0.20		

Table 2.4. Nitrogen components for synthetic grape must based on Bely *et al.* (1990) with a total YAN of 140 mg N/L.

Amino acids (mg/L)			
Tyrosine	8.40	Glutamine	235.67
Tryptophane	83.53	Alanine	67.67
Isoleucine	15.40	Valine	21.00
Aspartic acid	21.00	Methionine	14.47
Glutamic acid	56.00	Phenylalanine	17.73
Arginine	174.07	Serine	36.87
Leucine	22.40	Histidine	15.40
Threonine	35.47	Lysine	7.93
Glycine	8.40	Cysteine	6.07
Asparagine	24.73	Proline	286.07
Ammonium salt (mg/L)			
Ammonium chloride			214.67

Table 2.5. Recommended dosage of commercial yeast nutrients with corresponding YAN values and the treatment dosage used in the study.

Content	Yeast derived			Yeast derived with mineral salt				Mineral salt
	Y – 1	Y – 2	Y – 3	YM – 1	YM – 2	YM – 3	YM – 4	M – 1
Recommended dosage (mg/L)	300 – 600	300 – 600	200 – 400	300 – 500	300 – 400	200 – 400	300 – 500	100 – 500
Dosage YAN[1] (mg N/L)	10 – 20	9 – 19	4 – 7	36 – 60	45 – 60	26 – 52	43 – 72	21 – 106
Treatment dosage[2] (mg/L)	600	600	380	370	300	350	310	210

[1] Yeast assimilable nitrogen; [2] corresponding to 45mg N/L, or highest dosage and supplemented with DAP

4.2.4 Fermentation kinetics and chemical analysis

Fermentation kinetics were monitored through accumulated weight loss and yeast population dynamics by using flow cytometry as described above and plating. Flasks were weighed daily until weight loss was less than 0.1 g for three consecutive days. This was considered the end of fermentation. Total yeast population was determined for day 2, 4, 8 and the end of fermentation. For sequential fermentations, individual yeast populations were monitored through surface plating on day 4, 8 and at end of fermentation. Differential and selective agar media were utilized to distinguish between yeasts with similar colony morphology or non-detectable at dilution ranges for *S. cerevisiae*. For *T. delbrueckii*, Wallerstein Laboratory (WL) agar (Merck) was used throughout the fermentation. For *P. kluyveri* and *M. pulcherrima*, YPD agar and a selective carbon source agar medium (SCS-carbon source) was used (Kurtzman *et al.* 2011). Distinction for *P. kluyveri* was done on YPD agar (day 4 and 8) and SCS-xylitol (end of fermentation). For *M. pulcherrima*, distinction was done by plating on YPD agar (day 4) and SCS-mannitol (day 8 and end of fermentation). The major fermentation metabolites (glucose, fructose, ethanol and glycerol) were measured with the Wine-scan FT120 instrument (FOSS Analytical A/S, Hillerød, Denmark) with in-house calibrations specific for synthetic grape must and white grape must (Nieuwoudt *et al.* 2006).

2.2.5 Major volatile compound analysis and odour activity values (OAVs)

Major volatile compounds for the *M. pulcherrima* sequential yeast combination treatments in the synthetic and Chenin blanc must were measured by gas chromatography fitted with a flame ionisation detector (GC-FID) as described by Louw *et al.* (2009). The analysis was done using a

J&W DB-FFAP capillary GC column (Agilent, Little Falls, Wilmington, DE) (dimensions 60 m length × 0.32 mm i.d. × 0.5 µm f.t.). The samples were prepared following a liquid-liquid extraction protocol. Briefly, 5 mL of the sample (centrifuged beforehand) and 100 µL of the internal standard, 4-methyl-2-pentanol, were added to glass vials and were extracted with 1 mL diethyl ether in ultrasonic bath for 5 min. The vials were then cooled before being centrifuged for 3 minutes at 1789 g. The ether layer (top layer in vial) was added to GC-FID vials that contained sodium sulphate (dries the sample) and after mixing, the organic layer was added to the insert before clamping the GC-FID vial shut for analysis. To further understand the volatile esters measured and how they could contribute positively to wine sensory properties, the odour activity value (OAV) was calculated by dividing the measured concentration with the odour threshold for each compound (Table 2.6).

Table 2.6 Odour descriptors and thresholds of volatile esters that contribute positively to wine sensory properties.

Compound	Odour description	Threshold (mg/L)
Isoamyl acetate	Banana [1]	0.030 [4]
Ethyl hexanoate	Green apple, banana [2]	0.014 [5]
Ethyl octanoate	Pineapple, floral, pear [3]	0.005 [5]
Ethyl lactate	Buttery, fruity [1]	154.640 [6]
Diethyl succinate	Fruity, melon [1]	200.000 [6]
2-Phenylethyl acetate	Rose, honey [2]	0.250 [4]

[1] Louw *et al.* (2010); [2] Zea *et al.* (2007); [3] Jiang and Zhang (2018); [4] Guth (1997); [5] Ferreira *et al.* (2000); [6] Etievant (1991).

2.2.6 Statistical analysis

The nonlinear procedure (PROC NLIN) of SAS software (Version 9.4; SAS Institute Inc, Cary, USA) was used to fit a modified exponential function (Figure 2.1.a) on the accumulated weight loss data for single yeast *S. cerevisiae* and *T. delbrueckii* fermentations as well as the sequential fermentations. For single yeast *P. kluyveri* and *M. pulcherrima* the natural growth function (Figure 2.1.b) was used. The day where 50% of maximum weight loss was recorded (EC50 value) was then calculated (Figure 2.1.c). Statistical analysis on EC50 values, cell count and chemical analysis data was performed with XLSTAT version 2019 statistical software (Addinsoft, Boston, USA) using the one-way analysis of variance (ANOVA) at 95% accuracy level. Tukey's (HSD) test was used as comparison test when samples were significantly different after ANOVA ($P < 0.05$).

A $y = a\left(1 - e^{bt^2}\right)$

B $y = a(1 - e^{bt})$

C $EC_{50} = \sqrt{\frac{\log\left(1-\left(\frac{x}{a}\right)\right)}{b}}$

Figure 2.1 Formulas of interest for statistical analysis, modified exponential function (A), natural growth function (B) and function for EC50 value (C). Where y is the dependent variable (accumulated weight loss), a is the maximum value, e= 2.7188, b is the rate of increase/decrease, t is the time (day) and x is half of the maximum value.

2.3 Results

2.3.1 Commercial yeast nutrients

The YAN of the different nutrient formulations were determined and used to calculate the treatment dosages implemented in this study (Table 2.5). The treatment dosage had two requirements; firstly, it had to be within the manufacturer's recommended dosage range and secondly, the YAN value had to be the same for the different treatments. Only the yeast derivative and mineral salt (YM) and mineral salt (M) yeast nutrients met both requirements, while the yeast derived (Y) nutrients all had lower YAN values. Therefore, for the Y nutrient treatments, the highest recommended dosage was used and supplemented with DAP.

The amino acid and ammonia concentrations for the different nutrient treatments were also analysed and are shown in Table 2.7 and A2.1. The Y nutrient treatments had the highest concentration of amino acids, especially preferred, branched-chain and aromatic amino acids. However, they only contributed 5 mg N/L (yeast hulls nutrient) or 13 – 14 mg N/L (autolysate nutrients) of YAN (Table A2.1). The YM nutrients however, had low amino acid concentrations and only amino acids preferred by *S. cerevisiae* were detected (Ljungdahl and Daignan-Fornier 2012). Nutrient treatments YM1 and YM3 also had amino acid concentrations that were double than that of nutrient treatment YM2 and YM4. The major contributor to the YAN of these nutrients were ammonia and all the nutrients had similar concentrations of YAN. As expected, no amino acids were detected in the M nutrient treatment. This nutrient consists of ammonium sulphate (AS) salts and is the reason for the high ammonia concentrations and YAN value. Of note is that although the nutrients were calculated to contribute 45 mg N/L, the calculated YAN values ranged from 40 to 50 mg N/L.

Table 2.7 Summary of nutrient treatments based on chemical analysis, concentrations in mg/L and YAN in mg N/L. Amino acid groupings specific for *S. cerevisiae* (Ljungdahl and Daignan-Fornier 2012).

Content	Yeast derived			Yeast derived with mineral salt				Mineral salt
	Y – 1	Y – 2	Y – 3	YM – 1	YM – 2	YM – 3	YM – 4	M – 1
Preferred amino acids[1]	52.92	59.44	21.38	4.89	2.71	6.06	2.74	N/D[2]
Branched-chain and aromatic amino acids[3]	40.64	45.71	18.91	N/D	N/D	N/D	N/D	N/D
Other amino acids[4]	21.94	20.90	7.81	N/D	N/D	N/D	N/D	N/D
Total amino acids	115.50	126.05	48.10	4.89	2.71	6.06	2.74	N/D
Ammonia	1.17	0.39	1.09	49.80	55.84	60.96	53.59	54.53
Supplemented DAP	30.69	31.98	46.69	N/S[5]	N/S	N/S	N/S	N/S
Calculated YAN	39.18	40.38	44.59	41.46	46.16	50.79	44.31	44.85

[1] (Arg, Glu, Ala, Asp, Asn, Ser and Gln), [2] not detected, [3] (Val, Leu, Ile, Phe, Tyr and Trp), [4] (Thr, Gly, Met, Lys and His), [5] not supplemented.

2.3.2 Fermentations – Synthetic grape must

Fermentations were monitored through accumulated weight loss and yeast cell counts. The accumulated weight loss data was used to calculate the EC50 value which is the estimated day where 50% of maximum weight loss was recorded and represents the midpoint of fermentation. This value was used as an indication of the fermentation rate. For the single yeast fermentations, the maximum cell counts observed was used as indication of ability of the nutrient to support the growth of the different yeasts. The sequential culture fermentations had more complex yeast cell count data and were displayed as overall yeast populations. The last individual yeast cell counts recorded were used to evaluate to ability of nutrient to sustain the non-*Saccharomyces* component within the sequential fermentation. Major fermentation metabolites were quantified at the end of fermentation. Only observations that were significantly different by the Tukey (HSD) test at 5% level of significances are reported.

2.3.2.1 Single yeast fermentations

Among the single yeast fermentations, *S. cerevisiae* took 12 days to finish alcoholic fermentation, while *T. delbrueckii* took 14-16 days. The *P. kluyveri* and *M. pulcherrima* fermentations were sluggish and stopped after 14 days. The major fermentation metabolites measured are shown in

Table 2.8; no differences between the different treatments could be observed. The *M. pulcherrima* fermentation had the highest ethanol yield, while *P. kluyveri* had the lowest ethanol yield and the highest glycerol yield. *T. delbrueckii* had a similar ethanol yield as *S. cerevisiae* and the lowest glycerol yield.

Table 2.8 Average major fermentation metabolites for all treatments per single yeast fermentation.

	Yeast strain[1]			
Parameter	***S. cerevisiae***	***T. delbrueckii***	***P. kluyveri***	***M. pulcherrima***
Residual sugar (g/L)	3.38 ± 0.69 c	5.49 ± 1.85 c	126.49 ± 5.45 b	135.08 ± 5.54 a
Ethanol (% vol/vol)	12.13 ± 0.13 a	12.02 ± 0.17 a	4.20 ± 0.30 c	4.56 ± 0.36 b
Glycerol (g/L)	5.31 ± 0.08 a	4.84 ± 0.11 b	3.39 ± 0.22 c	2.09 ± 0.13 d
Ethanol yield (g/g)	0.49 ± 0.00 b	0.49 ± 0.00 b	0.45 ± 0.01 c	0.56 ± 0.02 a
Glycerol yield (mg/g)	27.00 ± 0.44 c	24.95 ± 0.53 d	46.18 ± 1.58 a	32.28 ± 2.09 b

[1] Means in same row with common letter are not significantly different (P > 0.05).

The EC50 value for all the yeasts is illustrated in Figure 2.2. Nutrient treatment Y2 resulted in the overall fastest fermentation for all the yeasts. Nutrient treatment Y2 also induced the fastest fermentation for *S. cerevisiae* and *M. pulcherrima* and was faster than nutrient treatment Y3 for these yeasts. Nutrient treatment Y2 and YM4 induced the fastest fermentation for *T. delbrueckii*, while Y3 was the fastest for *P. kluyveri*. The negative control resulted in the slowest fermentation for *S. cerevisiae* and *T. delbrueckii*, while YM2 and M1 resulted in the slowest fermentations for *P. kluyveri* and *M. pulcherrima* respectively. There were no differences between YM nutrient treatments for *S. cerevisiae*, *T. delbrueckii* and *M. pulcherrima*, while nutrient treatment YM3 and YM4 induced faster fermentations than YM2 for *P. kluyveri*.

All the yeasts reached maximum population on day four, except for *M. pulcherrima* that took eight days. The maximum cell counts for all the yeasts are illustrated in Figure 2.3. Nutrient treatments Y1, YM2 and YM3 induced the highest cell counts for *S. cerevisiae*. Nutrient treatment YM3 also induced the highest cell count for *T. delbrueckii* and was significantly higher than nutrient treatment YM2. For *P. kluyveri* and *M. pulcherrima*, nutrient treatment Y2 and the positive control induced the highest cell counts respectively. The negative control had the lowest cell for *S. cerevisiae* and *M. pulcherrima*. The negative control as well as nutrient treatment M1 induced the lowest cell counts

for *P. kluyveri*. Nutrient treatment Y2 induced the lowest cell count for *T. delbrueckii* and was significantly lower than nutrient treatment Y1.

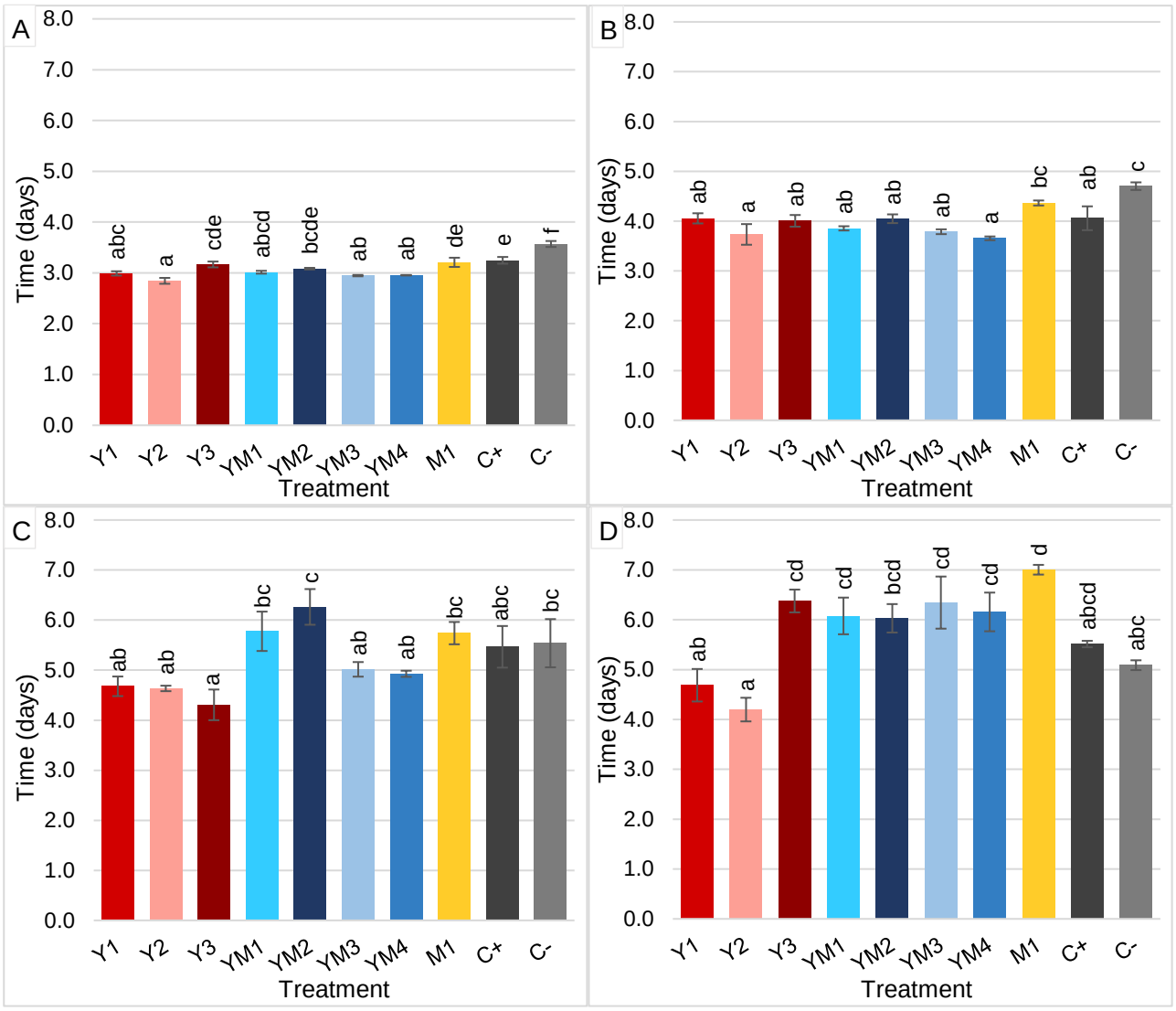

Figure 2.2 EC50 values for each single yeast fermentation; *S. cerevisiae* (A), *T. delbrueckii* (B), *P. kluyveri* (C) and *M. pulcherrima* (D). Results indicated are the mean of three biological repeats with ± standard deviation error bars. Means with common letter above are not significantly different (P > 0.05).

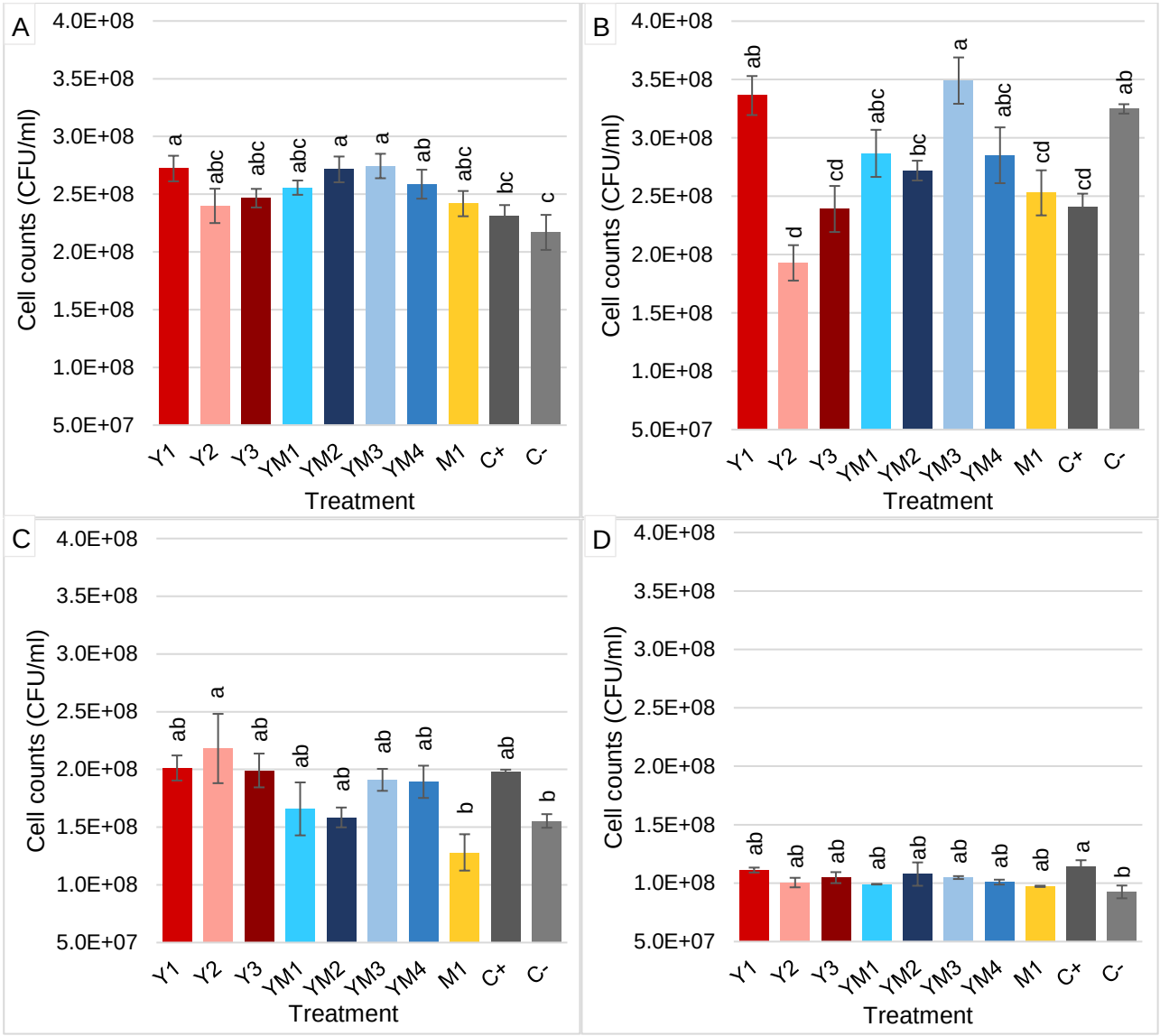

Figure 2.3 Maximum cell count for each single yeast fermentation; *S. cerevisiae* (A), *T. delbrueckii* (B), *P. kluyveri* (C) and *M. pulcherrima* (D). Results indicated are the mean of three biological repeats with ± standard deviation error bars. Means with common letter above are not significantly different (P > 0.05).

Overall a few observations are possible for fermentation speed and cell counts for different yeasts. For *S. cerevisiae*, the negative control had the slowest fermentation with the lowest cell count compared to treatments with higher YAN. Furthermore, although some of the nutrients slightly improved the fermentation speed, the cell counts for these treatments were not significantly different. A similar result was observed for both *P. kluyveri* and *M. pulcherrima*, where certain nutrients had improved fermentation speed while the cell counts were not significantly different. The only exception was nutrient treatment M1 for *P. kluyveri* that displayed the lowest cell count compared with treatment Y2. With regards to *T. delbrueckii*, the results indicate that nutrient treatment Y2 induced the fastest fermentation but the lowest cell count. The response of *T. delbrueckii* to nutrient Y2 shows

that a higher cell count does not result in faster fermentation. This was however not case for all nutrients, as nutrient treatment YM3 that had highest cell count did not induce fermentation speed significantly slower than nutrient Y2.

2.3.2.2 Sequential culture fermentations

The *T. delbrueckii* and *M. pulcherrima* sequential fermentations took 15-18 days to finish, while *P. kluyveri* sequential fermentations took 20-25 days. The major fermentation metabolite results show that there were no differences between the different treatments (Table 2.9). All the sequential fermentations were able to complete alcoholic fermentation. The data show that *M. pulcherrima* sequential fermentation had the highest ethanol yield, while the *P. kluyveri* sequential fermentation had the lowest ethanol yield together with the highest glycerol yield. *T. delbrueckii* sequential fermentation displayed the lowest glycerol yield.

Table 2.9 Average major fermentation metabolites for all treatments per sequential yeast combination.

Parameter	**Yeast strain[1]**		
	T. delbrueckii* with *S. cerevisiae	***P. kluyveri* with *S. cerevisiae***	***M. pulcherrima* with *S. cerevisiae***
Residual sugar (g/L)	6.10 ± 2.01 a	5.92 ± 0.88 a	5.46 ± 1.09 a
Ethanol (% vol/vol)	12.03 ± 0.18 b	11.86 ± 0.13 c	12.25 ± 0.09 a
Glycerol (g/L)	5.34 ± 0.12 c	5.72 ± 0.12 a	5.61 ± 0.08 b
Ethanol yield (g/g)	0.49 ± 0.00 b	0.48 ± 0.00 c	0.50 ± 0.00 a
Glycerol yield (mg/g)	27.54 ± 0.62 c	29.49 ± 0.64 a	28.82 ± 0.48 b

[1] Means in same row with common letter are not significantly different (P > 0.05).

The non-*Saccharomyces* yeasts reached different cell counts within the first 48 h, with *T. delbrueckii* (Figure 2.4.a) and *P. kluyveri* (Figure 2.5.a) reaching cell counts of 2.0 x 10^8 and 1.7 x 10^8 CFU/mL respectively and *M. pulcherrima* (Figure 2.6.a) only reaching 4.9 x 10^7 CFU/mL. The cell counts of *T. delbrueckii* started to decline after the inoculation of *S. cerevisiae*, while *P. kluyveri* and *M. pulcherrima* only started to decline after day four. Of interest is that some of the nutrient treatments for *P. kluyveri* sequential fermentations displayed decreased (Y3, YM4, M1 and negative control) or stable (Y1 and positive control) *S. cerevisiae* cell counts after the *P. kluyveri* cell count decreased. The last detectable cell count for both *P. kluyveri* and *M. pulcherrima* was on day eight, while *T. delbrueckii* persisted till the end of the fermentation.

Nutrient treatment Y1 and Y2, as well as all the YM nutrient treatments induced the fastest fermentations for the *T. delbrueckii* (Figure 2.4.b) sequential fermentations. Nutrient treatment Y2 also induced the fastest fermentation for *P. kluyveri* (Figure 2.5.b) and *M. pulcherrima* (Figure 2.6.b) sequential fermentations and was faster than the other Y nutrient treatments for these yeasts. Nutrient treatment M1 resulted in the slowest fermentation for *T. delbrueckii and P. kluyveri* sequential fermentations, while the two controls had the slowest fermentations for *M. pulcherrima* sequential fermentations. There were no differences between YM nutrients for *T. delbrueckii and P. kluyveri* sequential fermentations. For *M. pulcherrima* sequential fermentations, the YM nutrient treatments had two groupings, with YM1 and YM4 faster than YM2 and YM3.

The final cell counts for the different sequential fermentations were distinct for the different non-*Saccharomyces* yeasts. In the case of *T. delbrueckii*, a negative correlation between the final cell counts of non-*Saccharomyces* and *S. cerevisiae* was observed (Figure 2.4.c). The negative control had the highest *T. delbrueckii* and lowest *S. cerevisiae* cell count, while nutrient treatment YM2 had the lowest *T. delbrueckii* and highest *S. cerevisiae* cell count. The last detectable cell count of *P. kluyveri* appeared to have a positive correlation with the final *S. cerevisiae* cell count (Figure 2.5.c). Nutrient treatment YM1 had the highest final cell counts for both *P. kluyveri* and *S. cerevisiae*, while nutrient Y2 had the lowest for both yeasts. There was no clear pattern for the final cell counts for the *M. pulcherrima* sequential fermentations (Figure 2.6.c). It was the highest for the negative control, while nutrient treatment Y2 displayed the lowest (below 1 x 10^4 CFU/ml) cell count although it was not significantly different from other lowest value treatments. All the nutrient treatments except YM2 and YM3 and the two controls showed final *M. pulcherrima* cell counts lower than the original inoculation. The final *S. cerevisiae* cell count was highest for nutrient treatments Y1, Y2 and YM1 and the lowest for nutrient treatments Y3 and YM2.

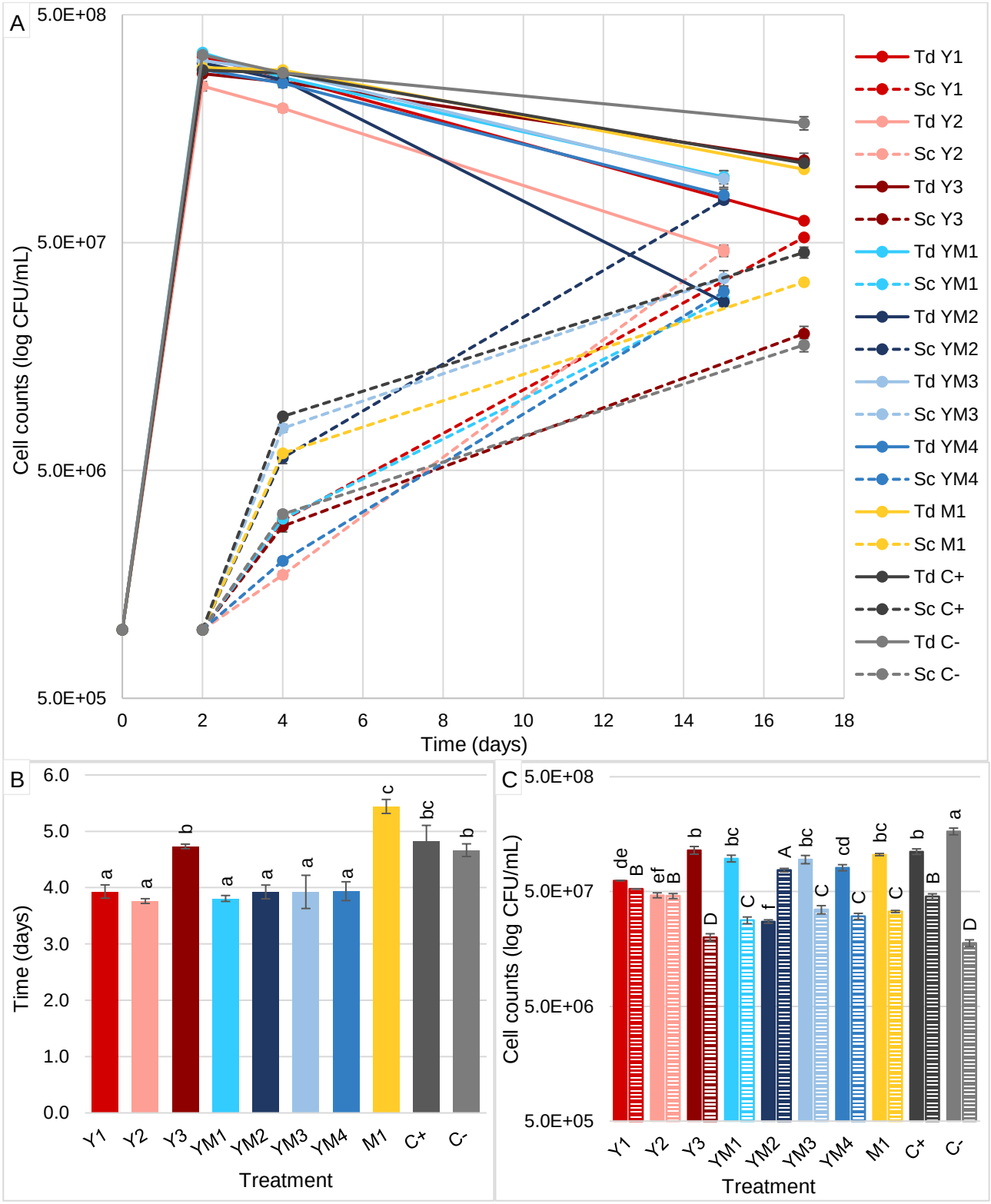

Figure 2.4 Fermentation kinetics for *T. delbrueckii* sequential fermentation in synthetic must. Population dynamics (A) for each treatment, *T. delbrueckii* (solid line) and *S. cerevisiae* (dashed line). EC50 values (B) and last cell count (C) for each treatment, *T. delbrueckii* (solid bars, lowercase letter) and *S. cerevisiae* (striped bars, uppercase letter). Results indicated are the mean of three biological repeats with ± standard deviation error bars. Means for each yeast with common letter above are not significantly different (P > 0.05).

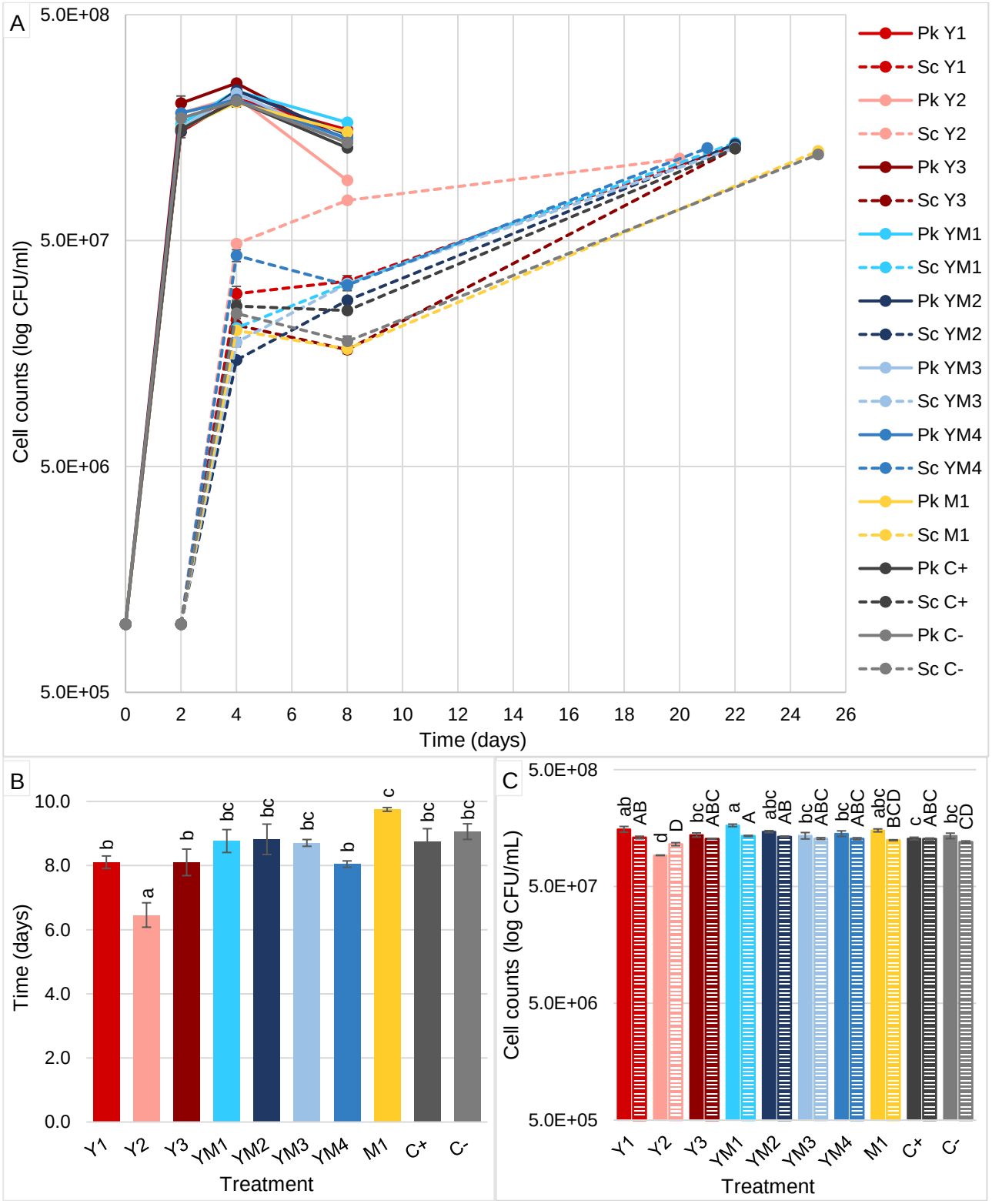

Figure 2.5 Fermentation kinetics for *P. kluyveri* sequential fermentation in synthetic must. Population dynamics (A) for each treatment, *P. kluyveri* (solid line) and *S. cerevisiae* (dashed line). EC50 values (B) and last cell count (C) for each treatment, *P. kluyveri* (solid bars, lowercase letter) and *S. cerevisiae* (striped bars, uppercase letter). Results indicated are the mean of three biological repeats with ± standard deviation error bars. Means for each yeast with common letter above are not significantly different (P > 0.05).

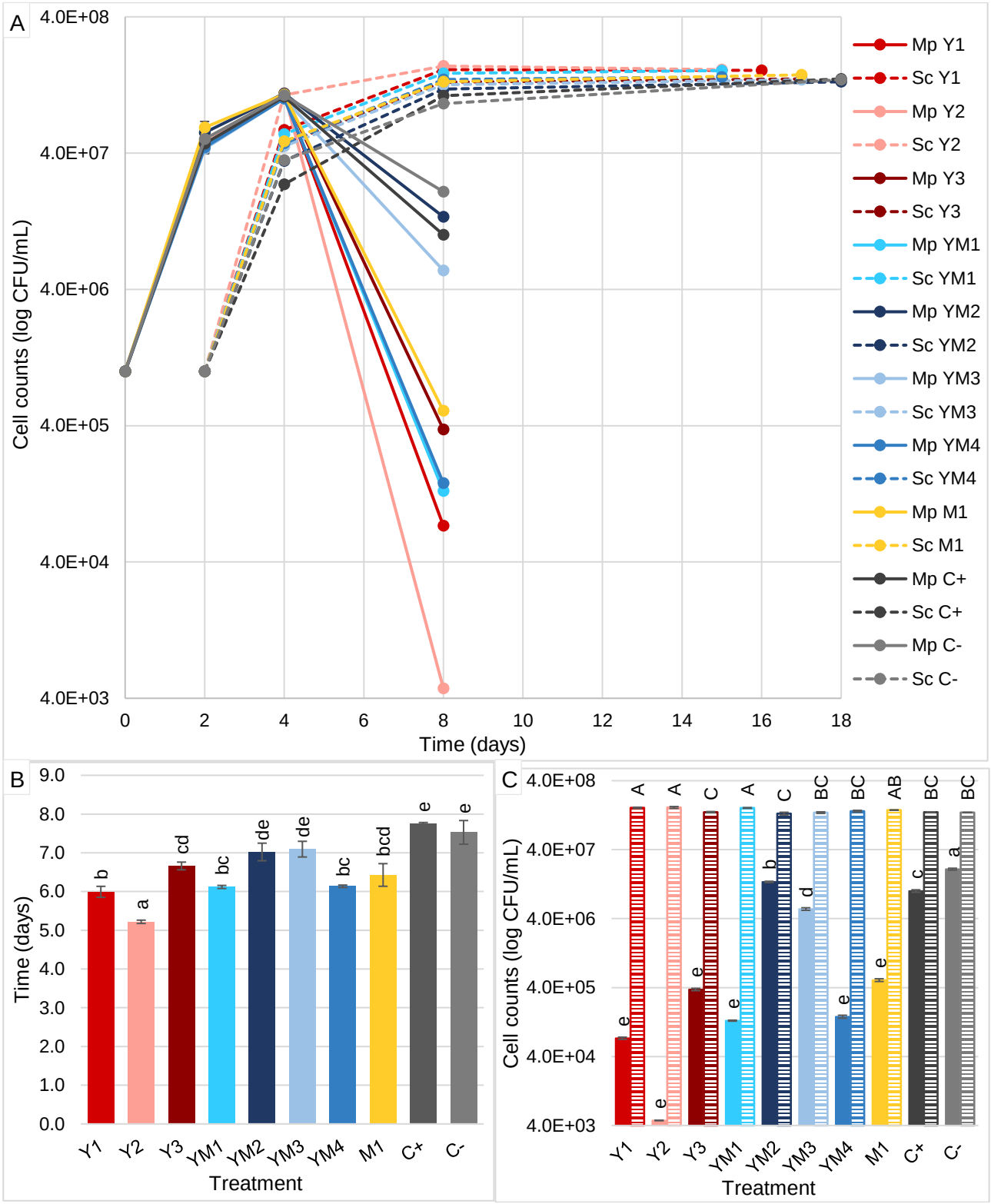

Figure 2.6 Fermentation kinetics for *M. pulcherrima* sequential fermentation in synthetic must. Population dynamics (A) for each treatment, *M. pulcherrima* (solid line) and *S. cerevisiae* (dashed line). EC50 values (B) and last cell count (C) for each treatment, *M. pulcherrima* (solid bars, lowercase letter) and *S. cerevisiae* (striped bars, uppercase letter). Results indicated are the mean of three biological repeats with ± standard deviation error bars. Means for each yeast with common letter above are not significantly different (P > 0.05).

2.3.3 Fermentations – Chenin blanc must

The sequential fermentation of *M. pulcherrima* with *S. cerevisiae* was selected for further investigation in real grape must, as this yeast combination had the most diverse response to the nutrient treatments. The fermentations in Chenin blanc took 13-15 days to finish and the average major fermentation metabolites measured show that there were no differences between the different treatments (Table 2.10). All fermentations reached dryness, with relatively low ethanol and high glycerol yield.

Table 2.10 Average major fermentation metabolites for all treatments of *M. pulcherrima* sequential fermentations in Chenin Blanc.

	Residual sugar (g/L)	**Ethanol (% vol/vol)**	**Glycerol (g/L)**	**Ethanol yield (g/g)**	**Glycerol yield (mg/g)**
Chenin blanc	1.83 ± 0.16	11.60 ± 0.10	8.14 ± 0.31	0.42 ± 0.00	37.32 ± 1.41

M. pulcherrima cell counts increased rapidly within the first 48 h, reaching cell counts of 1.0 x 10^8 CFU/mL (Figure 2.7.a). The cell counts for nutrient treatment YM3 and the positive control started to decrease after the inoculation of *S. cerevisiae*, while the rest of the fermentations only decreased after day four. The last detectable cell count for *M. pulcherrima* was on day eight. Nutrient treatment YM2 resulted in the fastest fermentation and was also faster than YM3 of the YM nutrient group (Figure 2.7.b). Within the Y nutrient treatment group, no differences were observed and nutrient treatment M1 resulted in the slowest fermentation. The final *M. pulcherrima* cell count for nutrient treatment M1 was the highest of all the treatments and along with nutrient treatment Y1 had a higher cell count than the controls (Figure 2.7.c). Nutrient treatment YM1 and YM4 had the lowest final *M. pulcherrima* cell count. All the treatments had cell counts lower than original inoculation. The final *S. cerevisiae* cell count was highest for the positive control and the lowest for nutrient treatment YM1. Within the Y and YM nutrient treatment groups, no differences were observed for the final *S. cerevisiae* cell count.

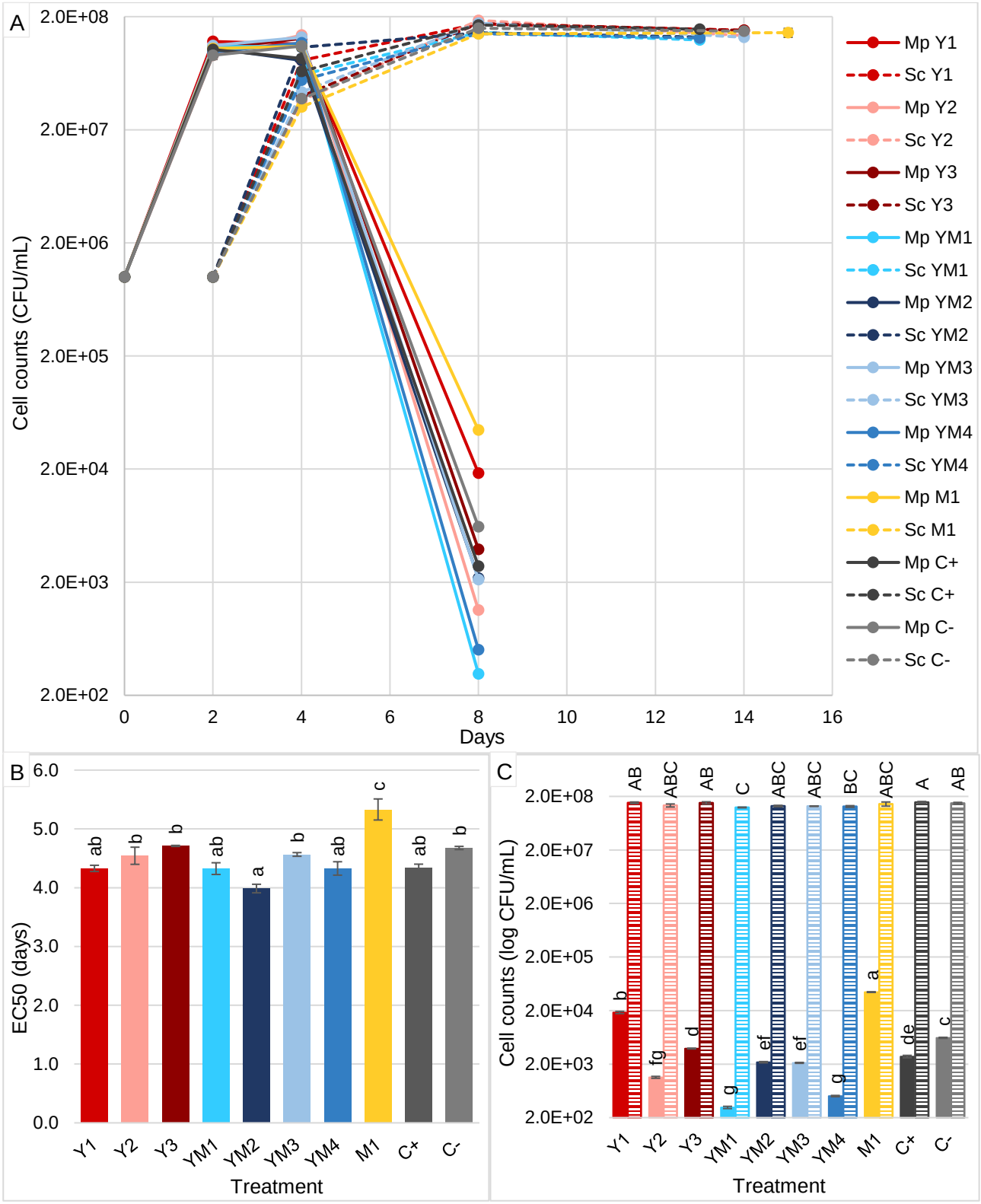

Figure 2.7 Fermentation kinetics for *M. pulcherrima* sequential fermentation in Chenin Blanc. Population dynamics (A) for each treatment, *M. pulcherrima* (solid line) and *S. cerevisiae* (dashed line). EC50 values (B) and last cell count (C) for each treatment, *M. pulcherrima* (solid bars, lowercase letter) and *S. cerevisiae* (striped bars, uppercase letter). Results indicated are the mean of three biological repeats with ± standard deviation error bars. Means for each yeast with common letter above are not significantly different (P > 0.05).

2.3.4 Major volatile compounds – must comparison

The major volatile compounds for each treatment measured are shown in Addendum A, for both synthetic (Table A2.3) and Chenin Blanc must (Table A2.4). Comparison between matrices for this study will focus on volatile compound families, instead of individual compounds. Acetic acid will also be compared as this is an important metabolite directly related to the volatile acidity of wine as well as a fermentation stress marker. Only observations that were significantly different by the Tukey (HSD) test at 5% level of significances are reported.

2.3.4.1. Volatile compound families and acetic acid

The concentrations of volatile compound families and acetic acid for each treatment of *M. pulcherrima* sequential fermentation in synthetic must and Chenin Blanc are displayed in Table 11 and Table 12 respectively. The aliphatic higher alcohols exceeded 350 mg/L in both matrices and were lower in synthetic fermentations (380 – 460 mg/L) than Chenin blanc fermentations (420 – 460 mg/L), with isoamyl alcohol contributing half of the total value in both matrices. The esters were also lower in synthetic fermentations (8.1 – 11.5 mg/L) than in Chenin blanc fermentations (12.5 – 15.5 mg/L), with isoamyl acetate contributing half of the total value in both matrices. The volatile fatty acids had similar ranges (8 – 11 mg/L) in both matrices. The concentration of acetic acid did not exceed 400 mg/L in both matrices and were higher in synthetic must fermentations (225 – 385 mg/L) than Chenin blanc fermentations (185 – 280 mg/L).

The trend observed for the volatiles for different treatments were different depending on the fermentation matrix. Only nutrient treatment YM2 had higher levels of aliphatic higher alcohols than Y1 and Y2 in synthetic must fermentations, while no differences were observed for Chenin blanc fermentations. In the synthetic must fermentations, the concentration of propanol was also significantly lower for nutrient treatment Y1 and Y2, and although not significant it was also lower in Chenin blanc fermentations. Nutrient treatment YM2 resulted in highest levels of esters in synthetic must and Chenin blanc fermentations. For the volatile fatty acids, Y2 had the highest level in synthetic must, while YM1 resulted in the highest level in Chenin blanc fermentations. The negative control had the lowest concentrations for both esters and volatile fatty acids in synthetic must

fermentations and was also the lowest in Chenin blanc fermentations along with nutrient treatment M1. Both controls had the highest levels of acetic acid in synthetic fermentations, while nutrient treatment Y2 and negative control were the highest in Chenin blanc fermentations. Nutrient treatment Y1 and YM2 respectively had the lowest acetic acid values in synthetic must and Chenin blanc fermentations.

Table 2.11 Concentrations (in mg/L) of volatile compound families and acetic acid for each treatment for *M. pulcherrima* sequential fermentation in synthetic must. Results indicated are the mean[1] of three biological repeats with ± standard deviation.

	Aliphatic higher alcohols	Esters	Volatile fatty acids	Acetic acid
Y1	377.58 ± 15.57 b	9.42 ± 0.36 c	8.98 ± 0.43 bc	225.91 ± 19.75 c
Y2	377.70 ± 31.88 b	9.58 ± 0.22 bc	10.54 ± 0.55 a	308.60 ± 28.93 abc
Y3	416.03 ± 15.51 ab	9.26 ± 0.21 cd	8.36 ± 0.21 bc	323.30 ± 26.66 ab
YM1	439.32 ± 17.69 ab	9.32 ± 0.08 cd	9.24 ± 0.48 abc	245.43 ± 21.85 bc
YM2	458.62 ± 17.12 a	11.35 ± 0.25 a	9.40 ± 0.03 ab	337.54 ± 13.23 ab
YM3	424.25 ± 10.28 ab	11.02 ± 0.10 ab	9.37 ± 0.12 abc	292.71 ± 26.75 abc
YM4	393.55 ± 12.41 ab	9.42 ± 0.28 c	8.60 ± 0.30 bc	263.00 ± 9.58 bc
M1	396.92 ± 24.91 ab	9.57 ± 0.65 bc	8.46 ± 0.47 bc	348.35 ± 16.23 ab
C+	413.62 ± 29.46 ab	8.78 ± 0.63 cd	8.11 ± 0.24 bc	383.28 ± 30.65 a
C-	379.08 ± 9.53 ab	8.07 ± 0.10 d	7.99 ± 0.08 c	377.39 ± 37.49 a

[1] Means in same column with common letter are not significantly different (P > 0.05).

Table 2.12 Concentrations (in mg/L) of volatile product families and acetic acid for each treatment for *M. pulcherrima* sequential fermentation in Chenin Blanc. Results indicated are the mean[1] of three biological repeats with ± standard deviation.

	Aliphatic higher alcohols	Esters	Volatile fatty acids	Acetic acid
Y1	437.53 ± 14.12 a	12.74 ± 0.23 bc	8.68 ± 0.23 c	251.11 ± 9.49 ab
Y2	447.14 ± 16.04 a	12.34 ± 0.12 c	9.72 ± 0.06 bc	276.12 ± 14.44 a
Y3	452.52 ± 15.12 a	12.92 ± 0.19 bc	8.76 ± 0.14 c	238.90 ± 18.83 ab
YM1	442.38 ± 21.75 a	14.89 ± 0.92 ab	10.98 ± 0.39 a	215.60 ± 10.44 bc
YM2	446.07 ± 9.16 a	15.39 ± 0.34 a	10.28 ± 0.22 ab	185.96 ± 9.81 c
YM3	461.58 ± 23.32 a	13.36 ± 0.45 bc	9.68 ± 0.27 bc	255.69 ± 14.07 ab
YM4	442.77 ± 14.26 a	14.72 ± 0.99 ab	9.75 ± 0.06 abc	241.11 ± 7.78 ab
M1	429.37 ± 12.56 a	12.25 ± 0.66 c	8.69 ± 0.38 c	248.45 ± 2.39 ab
C+	440.12 ± 11.9 a	13.13 ± 0.60 bc	9.5 ± 0.65 bc	263.79 ± 21.02 ab
C-	422.71 ± 11.6 a	12.33 ± 0.08 c	8.87 ± 0.31 c	279.02 ± 20.36 a

[1] Means in same column with common letter are not significantly different (P > 0.05).

2.3.4.2. Odour activity values (OAV)

The OAVs of the volatile esters measured for each treatment in synthetic must and Chenin blanc must were calculated. Of the six esters that could be detected in both matrices, ethyl lactate and diethyl succinate had OAV < 0.01 (data not shown). Isoamyl acetate had the highest OAVs followed by ethyl hexanoate and octanoate (Figure 2.8). 2-Phenylethyl acetate had the lowest OAV and was considerably lower in synthetic fermentations than the Chenin blanc fermentations. Overall, nutrient treatment YM2 had the highest OAV for all four esters in synthetic fermentations, while nutrient YM1 was the highest for Chenin blanc fermentations. The negative control had the lowest OAVs in synthetic must fermentations, while Y3 and the negative control were the lowest for Chenin blanc fermentations.

Nutrient treatment YM3 also had high OAVs for ethyl hexanoate and 2-phenyl acetate in synthetic must fermentations, while YM2 had the highest 2-phenylethyl acetate OAV in Chenin blanc. In the synthetic fermentations it appeared as though the YM nutrient group formed two sub-groups, with nutrient treatments YM2 and YM3 having higher ester OAVs than YM1 and YM4. This grouping was not observed for Chenin blanc fermentations, where nutrient treatment YM1 had generally higher OAVs and YM3 relatively lower than the other YM nutrient treatments. The OAVs of nutrient treatment M1 were fairly similar to most of the Y nutrient treatments in both matrices. Although there were no differences in the OAVs between treatments within the Y group in Chenin blanc fermentations, there were some differences for ethyl octanoate (Y1 higher than Y2) and 2-phenylethylacetate (Y2 higher than Y3) in synthetic fermentations.

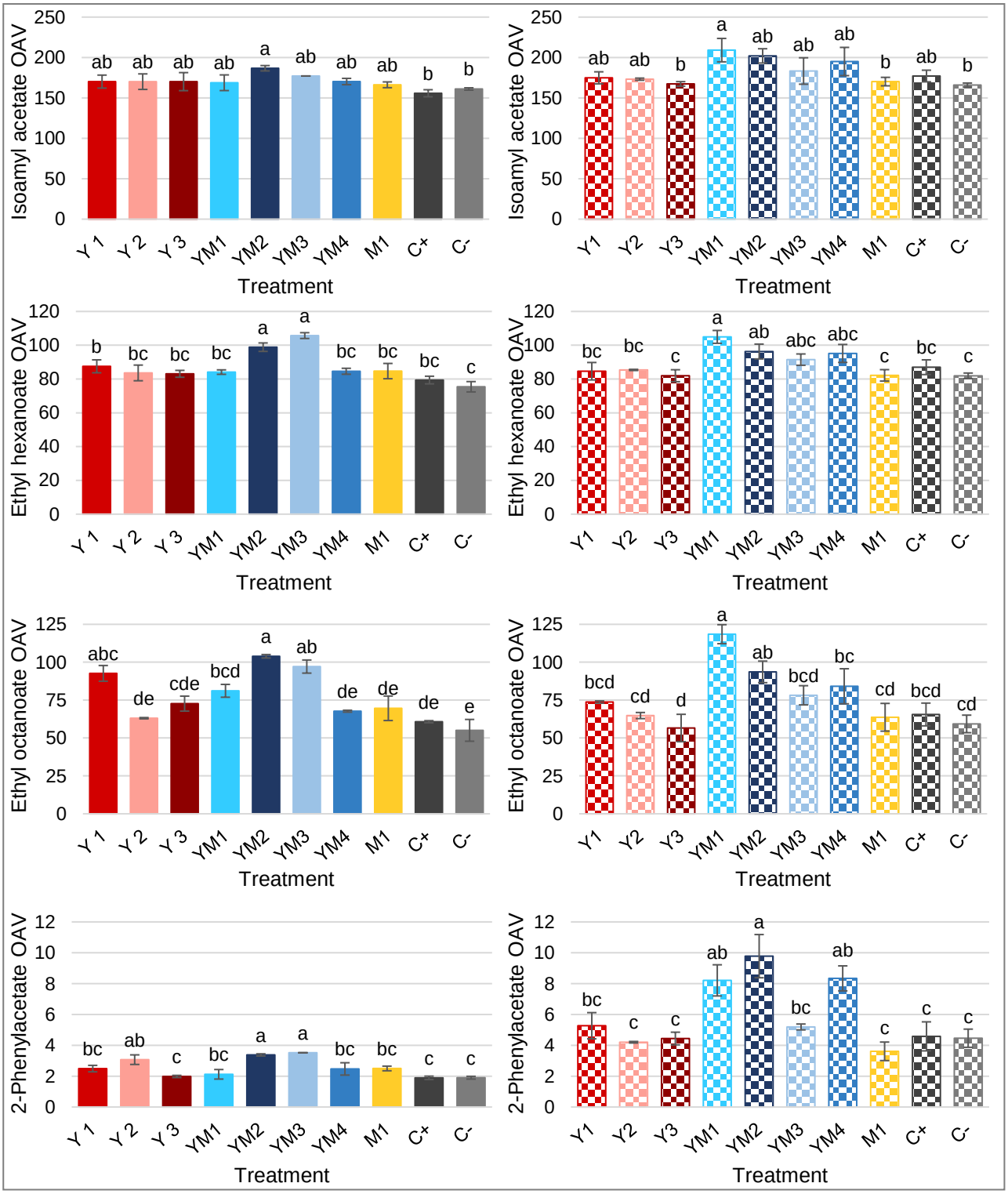

Figure 2.8 Odour activity values (OAV) for a few volatile compounds that could contribute to sensory properties of *M. pulcherrima* sequential fermentations in synthetic (solid, left) and Chenin Blanc (checker, right) for each treatment. Results indicated are the mean of three biological repeats with ± standard deviation error bars. Means with common letter are not significantly different (P > 0.05).

2.4 Discussion

Yeast nutrients are added to wine fermentations as a source of nitrogen to ensure a complete and efficient fermentation. Consequently, these nutrients have been formulated for *S. cerevisiae*. However, with non-*Saccharomyces* yeast commercially available it is important to know if these yeast nutrients can also be used to ensure an efficient non-*Saccharomyces* contribution to a wine fermentation. So, in line with the aims of this study a selection of nutrients and non-*Saccharomyces* yeasts were investigated.

2.4.1 Commercial yeast nutrients

Eight commercial yeast nutrients were investigated in this study. Most of the products contained yeast derivatives and can be divided into categories based on the extent that the yeast has been processed. Inactivated yeasts are whole yeast cells that are inactivated through heating. When these inactivated yeasts are further processed, either through mechanical or enzymatic activity, the cell walls degrade, and the content of the yeasts can be extracted (Jacob *et al.* 2019). The yeast content is known as yeast extract or yeast autolysate, while the cell walls are known as yeast hulls. Yeast autolysates, and to a lesser degree yeast hulls, are rich in amino acids, as shown in the results (Table 2.7/Section 3.3.1). The yeast nutrients (YM nutrients) that contained inactivated yeasts had negligible amino acid concentrations, which can be explained by a high ratio of mineral salt to yeast derivative or that the majority of the inactivated yeast are still intact. This is however only a hypothesis which should be further investigated. These nutrients are intended for *S. cerevisiae* and this is likely to be the reason that they are rich in amino acids preferred by *S. cerevisiae*. Some of the nutrients also contained thiamine, an essential co-factor for amino acid and carbohydrate catabolism (Li *et al.* 2010).

The mineral salt containing nutrients (YM and M nutrients) consisted of either DAP or AS, with only one nutrient (nutrient YM4) containing both. The use of DAP and AS in winemaking is well established and has been investigated in numerous studies (Martínez-Moreno *et al.* 2014; Andorrá *et al.* 2018; Pérez *et al.* 2018; Seguinot *et al.* 2018). A recent study by Andorrá *et al.* (2018) also found that when comparing DAP with AS, there were no significant differences for the production of

SO_2, although the use of DAP resulted in slightly elevated SO_2 levels. Furthermore, the study found that there were no differences for the other oenological parameters of kinetics for the different ammonium salts.

2.4.2 Fermentations – Synthetic grape must

One of the aims of this study was to investigate the use of commercial yeast nutrients to support the growth of non-*Saccharomyces* yeasts in sequential fermentations. To this end the nutrients were investigated in synthetic must for each non-*Saccharomyces* yeast in single yeast fermentations or sequential fermentations with *S. cerevisiae*. The latter was also used as reference yeast. The study was aimed to improve current understanding of yeast species preference and their possible competition for these nutrients. The yeasts used for in this study were selected based on recent studies in a larger non-*Saccharomyces* programme. Within this programme various previous studies have investigated their nitrogen and vitamin requirements as well as yeast interactions (De Koker 2015; Nutt 2018; Julies 2019; Prior *et al.* 2019). This study is the first to investigate commercial yeast nutrients.

2.4.2.1. Fermentation capability and major metabolites

The non-*Saccharomyces* single yeast fermentations fermented better in this study than previously reported by Prior *et al.* (2019) for these yeasts in similar medium. This could be due to the starvation of these yeasts in a nitrogen omitted medium before inoculation (performed by the latter authors) compared to rich medium used in this study for pre-culturing. For sequential fermentations, *T. delbrueckii* and *M. pulcherrima* had similar fermentation durations, while *P. kluyveri* took 5-8 days longer. The reason for extended fermentation duration for *P. kluyveri* sequential fermentation might have to do with the ability of *P. kluyveri* to compete effectively with *S. cerevisiae*. Indeed, in a study by Anfang *et al.* (2009), the authors found that when *P. kluyveri* was fermented with *S. cerevisiae* it could persist in the fermentations. Amongst the three non-*Saccharomyces* yeasts investigated, only *T. delbrueckii* was able to persist until the end of sequential fermentation. This can be expected, as *T. delbrueckii* is known to be the strongest fermenter among the non-*Saccharomyces* yeasts. The results also indicate that for the selected non-*Saccharomyces* sequential fermentations, a YAN of

140 mg N/L is sufficient to complete alcoholic fermentation in synthetic must with initial sugar concentration of 200 g/L.

In this study, no differences were observed for the major metabolites between the different treatments, in either single yeast or sequential fermentations. This could be due to the method of analysis used in this study, as the standard deviation of the average for all the treatments were within the prediction error of the metabolite quantified (Nieuwoudt *et al.* 2006). The result for different non-*Saccharomyces* single yeast fermentations, did however, indicate that the inverse correlation between glycerol and ethanol yields is not always perfectly conserved for the different non-*Saccharomyces* yeasts. The same trend was observed for sequential fermentations. The residual sugar of the fermentations that could complete alcoholic fermentation were between 3 and 6 g/L. Although not all the fermentations can be considered dry (less than 5 g/L) for white wine, it is possible that due to the fermentation volume and the manner in which the end of fermentation was determined that the fermentations could reach dryness should fermentation be continued for a day or two longer.

2.4.2.2. Fermentation kinetics

The results observed for *S. cerevisiae* single yeast fermentation are in agreement with previous studies that reported that increasing nitrogen content does indeed promote growth and fermentation speed for *S. cerevisiae* (Vilanova *et al.* 2007; Torrea *et al.* 2011). This is however the first report comparing different nutrient treatments (with same YAN value) for non-*Saccharomyces* single yeast and sequential fermentations. The results indicated that for the stronger fermenters (*S. cerevisiae* and *T. delbrueckii*) the negative control had the longest onset of fermentation, whereas for the weaker fermenters (*P. kluyveri* and *M. pulcherrima*) this was not the true. This could be due to their different nutrient utilisation and metabolic requirements; however, this hypothesis requires further investigation. The results further indicated that for all the single yeast fermentations, treatment Y2 resulted in overall fastest fermentation, but not always higher cell counts. Furthermore, some nutrients, especially the Y nutrients, benefited both non-*Saccharomyces* and *S. cerevisiae* and could thus result in nutrient competition during sequential fermentation. The Y nutrients are rich in amino

acids, which could be the reason for their ability to improve the onset of fermentation. This hypothesis require further investigation, as previous studies had found that nitrogen source, amino acids or ammonium, did not improve fermentation duration for *S. cerevisiae* (Torrea *et al.* 2011; Seguinot *et al.* 2018).

For the *T. delbrueckii* and *P. kluyveri* sequential fermentations there did not appear to be a link between cell count of either non-*Saccharomyces* or *S. cerevisiae* and the fermentation speed. However, for *M. pulcherrima* sequential fermentations, it appeared that higher final M. pulcherimma cell counts correlated with slower fermentations. Nutrient treatment M1, that induced the slowest fermentation for both *T. delbrueckii* and *P. kluyveri* sequential fermentations also induced slow fermentations for the single yeast fermentations, along with *S. cerevisiae*. This could indicate that the unfavourable effect of the nutrient on the fermentation speed in sequential fermentations could be accumulative.

The ability of the different nutrients to promote and sustain non-*Saccharomyces* yeasts in sequential cultures appeared to be specific for different yeasts. As far as could be determined, this is the first study to investigate numerous nutrient treatments (with same YAN) for different non-*Saccharomyces* yeasts sequential fermentation and observe patterns for the cell counts of different species. For the *T. delbrueckii* sequential fermentations there seemed to be direct competition of different nutrients as the final cell count for *T. delbrueckii* and *S. cerevisiae* showed a negative correlation. The competition for nutrients between these yeasts is further corroborated in the study by Prior *et al.* (2019), where the authors found that *T. delbrueckii* had similar amino acid preference as *S. cerevisiae* and that it was able to consume most of the available amino acids within the first 48 h of fermentation. The opposite was observed for *P. kluyveri* sequential fermentations, where a positive correlation was observed for final *P. kluyveri* and *S. cerevisiae* cell count. It has already been mentioned that this yeast is able to compete with *S. cerevisiae* (Anfang *et al.* 2009) and could hypothetically also inhibit *S. cerevisiae* to some degree when cell death occurs. This hypothesis is further validated in the results where early *P. kluyveri* cell death also negatively affected the *S. cerevisiae* cell count for some of the nutrient treatments. No apparent pattern between

M. pulcherrima and *S. cerevisiae* final cell count was observed. It is however important to note nutrient treatments YM2 and YM3 were able to significantly improve the final *M. pulcherrima* cell count. As a similar result was not observed for single *M. pulcherrima* fermentations this is likely due to an accumulative effect of the interaction with the nutrient and/or *S. cerevisiae*. With regards to *P. kluyveri* and *M. pulcherrima*, they both consume low concentrations of amino acids within the first 48 h (Prior *et al.* 2019), therefore the results observed is more likely due to the interaction between yeast species than direct competition for nitrogenous compounds.

2.4.3 Comparing synthetic must with Chenin blanc

Another aim of this study was to further investigate the use of commercial yeast nutrients to support the growth of one of the non-*Saccharomyces* yeasts in sequential fermentation in Chenin blanc grape must. Although the synthetic grape must was formulated to resemble a grape must (Henschke and Jiranek 1993), it is less complex than grape must and synthetic grape must studies should always be followed by the same investigation in real must. *M. pulcherrima* was selected for these further studies as the results in synthetic must indicated that the yeast had the most diverse response to the different nutrient supplementations. This yeast also had weak growth and could not compete as well as the other non-*Saccharomyces* yeasts with *S. cerevisiae*. It is possible that nutrients might improve the growth and persistence of *M. pulcherrima* in co-inoculated fermentations with *S. cerevisiae*.

2.4.3.1 Fermentations

The fermentations in Chenin blanc were faster, with lower ethanol yield and higher glycerol yield than the synthetic fermentations. The *M. pulcherrima* population within the first 48 h was also more than double in the Chenin blanc fermentaions than in the synthetic fermentations. Unlike for the fermentations in synthetic must, there was no obvious links between cell count of either *M. pulcherrima* or *S. cerevisiae* and the fermentation speed in Chenin blanc fermentations. This is most likely due to the differences in the two matrices. The only exception appeared to be for nutrient treatment M1, where the highest final *M. pulcherrima* cell count coincided with slowest fermentation. Similar to the synthetic fermentations, there was no apparent pattern between the *M. pulcherrima*

and *S. cerevisiae* final cell count. The final *M. pulcherrima* cell counts were also lower in the Chenin blanc fermentations. Additionally, the ability of nutrient treatments YM2 and YM3 to improve the final *M. pulcherrima* cell count was only observed within the YM nutrient treatments in Chenin blanc. It appeared that in Chenin blanc, nutrient treatments M1 and Y1 had more pronounced effect on the final *M. pulcherrima* final cell count. Overall, this indicates that although some results were similar in the two different matrices, there were still differences observed due to the complexity of Chenin in comparison to the synthetic must.

2.4.3.2 Major volatile compounds

The different volatile compound families can contribute to wines differently. For example, when aliphatic higher alcohols are below 350 mg/L they can impart complexity to the sensory properties of a wine (Swiegers *et al.* 2005; Esccribano *et al.* 2018). However, when the concentration exceeds this value, as is the case in this study, they can impart negatively on wines and also mask some of the fruity aromas (De-la-Fuente-Blanco *et al.* 2017). Other compounds such as volatile fatty acids and acetic acid can also be detrimental to wine if they are above 20 mg/L and 700 mg/L respectively (Swiegers *et al.* 2005; Englezos *et al.* 2018). However, in this study both the volatile fatty acids and acetic acid were below these detrimental threshold values.

For the higher alcohols, propanol has been identified as a marker for nitrogen source type, with higher amino acid concentrations resulting in lower propanol concentrations than ammonium (Seguinot *et al.* 2018). Indeed, in the synthetic must fermentations, and to a lesser degree in Chenin blanc fermentations, nutrients Y1 and Y2 that had the highest amino acid concentrations, resulted in lower propanol concentrations when compared to the nutrients with high ammonium concentrations. The formation of esters has been found to be directly proportional to the amino acid concentration in must for *S. cerevisiae* (Garde-Cerdán and Ancín-Azpilicueta 2008). However, in the current study, nutrient treatments of the YM group had higher ester concentrations in both synthetic must and Chenin blanc fermentations when compared to the other nutrient treatments. This nutrient category (YM nutrient treatments) contained almost negligible quantities of amino acids (0.23 – 0.66 mg N/L). This disagreement in the results could be due to the interaction between yeast species

and/or nutrients, however this is only a hypothesis that require further investigation. It is also possible that ester concentrations are not related to the amino acid concentrations, as has been shown for *S. cerevisiae* (Crépin *et al.* 2017).

With regard to the ester levels and the possible sensory perception, the chemical data indicated that nutrient YM2 resulted in the highest concentrations of esters and would thus have the highest fruity attributed volatiles. However, the OAVs indicate that although nutrient treatment YM2 had the highest OAV for all the esters in synthetic fermentations, for Chenin blanc fermentations YM1 has the highest. This is likely due to higher ethyl hexanoate and octanoate OAV in Chenin blanc that have very low odour thresholds. For the synthetic fermentation there appeared to be a link between nutrient treatments with higher final *M. pulcherrima* cell counts and higher concentrations of desirable esters, at least as far as the YM nutrients treatments are considered. This same trend was not observed for the Chenin blanc fermentations. This could mean that the higher cell densities of *M. pulcherrima* did not necessarily contribute to increased esters, or that additional factors are influencing the production of these esters in Chenin blanc. It is important to note that the OAV of a compound can vary greatly depending on the matrix as well as the concentrations of other compounds and can only be used as an estimation. Additional sensory evaluation would be required to confirm OAV results.

2.5 Conclusion

This is the first study that investigated the use of complex yeast nutrients for non-*Saccharomyces* wine yeasts. From the nitrogen content of the yeast nutrients it is clear that they were formulated for *S. cerevisiae*. The results showed that the nutrients had a greater effect on the onset of fermentation than on the growth of the yeasts for single fermentations and that one nutrient (nutrient treatment Y2) was preferred by all the yeasts. It was also the first time that nitrogen supplementation at the same level but with different content was investigated for non-*Saccharomyces* wine yeast sequential fermentations. The results showed that nutrient selection can influence how well the growth of non-*Saccharomyces* yeasts are sustained in sequential fermentations. When the nutrients were further investigated in Chenin blanc must for *M. pulcherrima* sequential fermentation clear

differences were observed for different matrices. The fermentations in Chenin blanc were faster, with lower final *M. pulcherrima* cell counts and different nutrients better supported the growth of *M. pulcherimma* than in synthetic must fermentations. Therefore, synthetic must is not a true representative of how these nutrients might influence non-*Saccharomyces* in real grape must. Furthermore, nutrient selection can also greatly influence sensory properties of wine, however this should be further investigated and confirmed with sensory evaluation.

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Appendix A

Chapter 2
Additional Tables

Chapter 3. Appendix A: Additional tables

Table A2.1. Chemical analysis results for nutrient treatments, concentrations in mg/L, FAN and YAN in mg N/L. Amino acid groupings specific for *S. cerevisiae* (Ljungdahl and Daignan-Fornier 2012).

Content	Yeast derived			Yeast derived with mineral salt				Mineral salt
	Y – 1	Y – 2	Y – 3	YM – 1	YM – 2	YM – 3	YM – 4	M – 1
Arginine	5.85	7.90	2.48	2.03	1.58	1.67	1.35	N/D[1]
Glutamic acid	14.73	17.98	7.76	1.86	1.14	2.68	1.38	N/D
Alanine	17.09	12.76	6.73	1.00	N/D	1.72	N/D	N/D
Aspartic acid	4.14	6.56	1.79	N/D	N/D	N/D	N/D	N/D
Asparagine	4.94	5.45	1.24	N/D	N/D	N/D	N/D	N/D
Serine	4.98	5.62	1.38	N/D	N/D	N/D	N/D	N/D
Glutamine	1.17	3.18	N/D	N/D	N/D	N/D	N/D	N/D
Preferred amino acids	52.92	59.44	21.38	4.89	2.71	6.06	2.74	N/D
Valine	8.14	8.61	3.35	N/D	N/D	N/D	N/D	N/D
Leucine	14.51	15.21	6.28	N/D	N/D	N/D	N/D	N/D
Isoleucine	6.92	7.60	3.06	N/D	N/D	N/D	N/D	N/D
Phenylalanine	6.47	7.08	3.13	N/D	N/D	N/D	N/D	N/D
Tyrosine	2.70	5.25	1.87	N/D	N/D	N/D	N/D	N/D
Tryptophan	1.91	1.96	1.21	N/D	N/D	N/D	N/D	N/D
Branched-chain and aromatic amino acids	40.64	45.71	18.91	N/D	N/D	N/D	N/D	N/D
Threonine	4.73	5.69	1.73	N/D	N/D	N/D	N/D	N/D
Glycine	3.76	4.03	1.32	N/D	N/D	N/D	N/D	N/D
Methionine	2.17	2.00	1.10	N/D	N/D	N/D	N/D	N/D
Lysine	8.74	6.86	3.66	N/D	N/D	N/D	N/D	N/D
Histidine	2.54	2.32	N/D	N/D	N/D	N/D	N/D	N/D
Other amino acids	21.94	20.90	7.81	N/D	N/D	N/D	N/D	N/D
Ammonia	1.17	0.39	1.09	49.80	55.84	60.96	53.59	54.53
Calculated FAN	12.98	13.76	5.29	0.50	0.23	0.66	0.24	N/D
Calculated YAN	13.94	14.08	6.19	41.46	46.16	50.79	44.31	44.85

[1] Not detected

Table A2.2. Chemical analysis results for different musts, concentrations in mg/L, FAN and YAN in mg N/L. Amino acid groupings specific for *S. cerevisiae* (Ljungdahl and Daignan-Fornier 2012).

Content	Synthetic (YAN)	Chenin blanc (YAN)
Arginine	174.66 (14.04)	400.63 (32.21)
Glutamic acid	56.04 (5.33)	63.65 (6.06)
Alanine	67.72 (10.65)	154.71 (24.32)
Aspartic acid	20.55 (2.16)	25.92 (2.73)
Asparagine	24.75 (2.62)	12.10 (1.28)
Serine	36.43 (4.85)	51.33 (6.84)
Glutamine	235.84 (22.60)	81.91 (7.85)
Preferred amino acids	615.99 (62.25)	790.25 (81.29)
Valine	20.55 (2.46)	35.99 (4.30)
Leucine	22.42 (2.39)	43.51 (4.65)
Isoleucine	14.94 (1.60)	17.77 (1.90)
Phenylalanine	17.28 (1.47)	24.66 (2.09)
Tyrosine	8.41 (0.65)	10.45 (0.81)
Tryptophan	83.59 (5.73)	6.70 (0.46)
Branched-chain and aromatic amino acids	167.19 (14.30)	139.08 (14.21)
Threonine	35.03 (4.12)	103.20 (12.13)
Glycine	8.41 (1.57)	8.15 (1.52)
Methionine	14.48 (1.36)	4.06 (0.38)
Lysine	7.94 (0.76)	9.70 (0.93)
Histidine	14.94 (1.35)	54.29 (4.90)
Other amino acids	80.80 (9.16)	179.40 (19.86)
Ammonia	72.44 (56.25)	42.41 (34.88)
Calculated FAN	85.72	115.37
Calculated YAN	141.97	150.24

Table A2.3. Major volatile results for different treatments in synthetic must, concentration in mg/L.

	Yeast derived			Yeast derived with mineral salt				Mineral salt	Controls	
	Y – 1	**Y – 2**	**Y – 3**	**YM – 1**	**YM – 2**	**YM – 3**	**YM – 4**	**M – 1**	**C+**	**C-**
Higher alcohols										
Propanol	71.39 ± 8.12 de	47.58 ± 5.20 e	102.73 ± 8.01 abcd	105.1 ± 8.39 abcd	128.27 ± 5.54 a	119.76 ± 10.31 ab	91.28 ± 5.66 bcd	109.74 ± 9.76 abc	123.33 ± 19.91 ab	76.64 ± 3.26 cde
Butanol	0.62 ± 0.02 ab	0.79 ± 0.01 a	0.67 ± 0.08 ab	0.67 ± 0.02 ab	0.77 ± 0.07 a	0.69 ± 0.08 ab	0.64 ± 0.01 ab	0.65 ± 0.05 ab	0.51 ± 0.03 b	0.57 ± 0.04 b
Isoamyl alcohol	223.84 ± 5.58 abc	233.96 ± 14.6 abc	227.01 ± 4.66 abc	241.39 ± 2.56 ab	241.44 ± 3.94 a	219.51 ± 5.78 abc	220.85 ± 10.53 abc	207.82 ± 7.00 abc	198.17 ± 24.93 c	201.92 ± 4.17 bc
Isobutanol	81.73 ± 2.64 a	95.36 ± 14.12 a	85.62 ± 5.81 a	92.16 ± 6.72 a	88.14 ± 9.15 a	84.29 ± 5.16 a	80.78 ± 6.78 a	78.72 ± 11.92 a	105.08 ± 20.54 a	90.13 ± 10.68 a
2-Phenylethanol	72.57 ± 0.08 bc	97.00 ± 2.97 a	66.81 ± 0.84 c	68.42 ± 0.43 bc	78.54 ± 2.29 bc	70.67 ± 2.36 bc	69.84 ± 6.60 bc	67.54 ± 5.12 c	72.05 ± 3.78 bc	80.37 ± 1.07 b
Esters										
Ethyl acetate	50.2 ± 4.78 a	51.88 ± 2.11 a	61.74 ± 3.11 a	63.23 ± 13.27 a	81.47 ± 3.57 a	81.19 ± 9.75 a	62.58 ± 5.46 a	71.47 ± 11.79 a	78.99 ± 7.60 a	75.16 ± 13.69 a
Isoamyl acetate	5.11 ± 0.24 ab	5.10 ± 0.29 ab	5.10 ± 0.10 ab	5.06 ± 0.00 ab	5.6 ± 0.11 a	5.31 ± 0.34 ab	5.11 ± 0.29 ab	4.99 ± 0.12 ab	4.67 ± 0.13 b	4.83 ± 0.04 b
Ethyl hexanoate	1.23 ± 0.05 ab	1.17 ± 0.06 ab	1.16 ± 0.03 ab	1.18 ± 0.02 ab	1.38 ± 0.04 a	1.38 ± 0.14 a	1.18 ± 0.02 ab	1.19 ± 0.06 ab	1.11 ± 0.03 b	1.06 ± 0.04 b
Ethyl lactate	1.72 ± 0.09 bc	2.17 ± 0.15 ab	1.93 ± 0.21 abc	1.88 ± 0.05 abc	2.53 ± 0.16 a	2.03 ± 0.13 abc	1.86 ± 0.13 bc	2.25 ± 0.23 ab	2.16 ± 0.35 ab	1.39 ± 0.12 c
Ethyl octanoate	0.43 ± 0.06 ab	0.35 ± 0.05 b	0.36 ± 0.02 b	0.41 ± 0.02 ab	0.52 ± 0.01 a	0.43 ± 0.08 ab	0.34 ± 0.00 b	0.35 ± 0.04 b	0.30 ± 0.00 b	0.28 ± 0.04 b
Diethyl succinate	0.32 ± 0.02 b	0.28 ± 0.03 bc	0.20 ± 0.02 cde	0.26 ± 0.03 bcd	0.47 ± 0.03 a	0.51 ± 0.02 a	0.31 ± 0.04 b	0.27 ± 0.01 bcd	0.16 ± 0.03 de	0.14 ± 0.02 e
2-Phenylacetate	0.62 ± 0.05 bc	0.77 ± 0.08 ab	0.49 ± 0.02 c	0.53 ± 0.00 bc	0.85 ± 0.04 a	0.88 ± 0.02 a	0.62 ± 0.08 bc	0.62 ± 0.1 bc	0.47 ± 0.03 c	0.48 ± 0.02 c

Table A2.3. Continued.

	Yeast derived			Yeast derived with mineral salt				Mineral salt	Controls	
	Y – 1	**Y – 2**	**Y – 3**	**YM – 1**	**YM – 2**	**YM – 3**	**YM – 4**	**M – 1**	**C+**	**C-**
Volatile acids										
Propionic acid	1.64 ± 0.04 c	2.18 ± 0.17 ab	1.79 ± 0.12 bc	1.91 ± 0.09 abc	2.36 ± 0.02 a	1.97 ± 0.12 abc	1.76 ± 0.00 bc	2.06 ± 0.16 abc	2.06 ± 0.23 abc	1.61 ± 0.18 c
Isobutyric acid	0.67 ± 0.05 b	0.96 ± 0.10 a	0.67 ± 0.02 b	0.68 ± 0.04 b	0.53 ± 0.00 bc	0.48 ± 0.02 c	0.60 ± 0.01 bc	0.52 ± 0.05 bc	0.55 ± 0.04 bc	0.66 ± 0.04 b
Butyric acid	1.19 ± 0.05 ab	1.38 ± 0.05 a	1.08 ± 0.03 bc	1.16 ± 0.03 bc	1.21 ± 0.03 ab	1.12 ± 0.12 bc	1.16 ± 0.01 bc	1.05 ± 0.05 bc	1.1 ± 0.07 bc	0.96 ± 0.03 c
Isovaleric acid	0.78 ± 0.06 abcd	0.88 ± 0.08 ab	0.74 ± 0.01 bcd	0.85 ± 0.12 abc	0.59 ± 0.01 cd	0.57 ± 0.07 d	0.70 ± 0.02 bcd	0.64 ± 0.11 cd	0.63 ± 0.05 cd	0.98 ± 0.02 a
Valeric acid	0.50 ± 0.02 a	0.40 ± 0.05 a	0.42 ± 0.04 a	0.58 ± 0.04 a	0.48 ± 0.06 a	0.43 ± 0.04 a	0.49 ± 0.02 a	0.47 ± 0.07 a	0.45 ± 0.04 a	0.42 ± 0.04 a
Hexanoic acid	1.74 ± 0.17 ab	1.9 ± 0.24 a	1.55 ± 0.15 ab	1.61 ± 0.13 ab	1.68 ± 0.05 ab	1.73 ± 0.24 ab	1.52 ± 0.11 ab	1.55 ± 0.15 ab	1.28 ± 0.16 b	1.38 ± 0.10 ab
Octanoic acid	1.49 ± 0.15 ab	1.74 ± 0.15 a	1.24 ± 0.11 ab	1.49 ± 0.25 ab	1.52 ± 0.03 ab	1.49 ± 0.21 ab	1.41 ± 0.16 ab	1.26 ± 0.08 ab	1.02 ± 0.18 b	1.15 ± 0.14 b
Decanoic acid	1.13 ± 0.14 a	1.09 ± 0.05 ab	0.87 ± 0.01 bcd	0.97 ± 0.05 abcd	1.02 ± 0.00 abc	1.02 ± 0.11 abc	0.96 ± 0.02 abcd	0.91 ± 0.07 abcd	0.77 ± 0.07 d	0.83 ± 0.03 cd
Acetic acid	225.91 ± 19.75 d	308.6 ± 28.93 abcd	323.3 ± 26.66 abcd	245.43 ± 21.85 bcd	337.54 ± 13.23 abc	273.87 ± 2.94 abcd	244.73 ± 27.00 cd	379.28 ± 45.71 a	373.28 ± 40.65 ab	377.39 ± 37.49 a

Table A2.4. Major volatile results for different treatments in Chenin blanc, concentration in mg/L.

	Yeast derived			Yeast derived with mineral salt				Mineral salt	Controls	
	Y – 1	**Y – 2**	**Y – 3**	**YM – 1**	**YM – 2**	**YM – 3**	**YM – 4**	**M – 1**	**C+**	**C-**
Higher alcohols										
Propanol	86.65 ± 12.01 ab	86.83 ± 5.95 ab	86.94 ± 2.71 ab	105.77 ± 2.68 a	89.4 ± 3.27 ab	104.03 ± 6.26 a	92.53 ± 9.66 ab	103.12 ± 9.00 a	101.65 ± 2.39 ab	77.20 ± 3.18 b
Butanol	1.11 ± 0.10 b	1.16 ± 0.03 ab	1.10 ± 0.01 b	1.35 ± 0.07 a	1.22 ± 0.03 ab	1.25 ± 0.08 ab	1.23 ± 0.1 ab	1.30 ± 0.02 ab	1.28 ± 0.03 ab	1.22 ± 0.04 ab
Isoamyl alcohol	237.85 ± 12.04 a	250.47 ± 7.77 a	228.06 ± 12.08 a	244.14 ± 13.07 a	261.66 ± 19.05 a	247.34 ± 7.57 a	245.19 ± 14.03 a	229.17 ± 3.77 a	234.92 ± 10.24 a	236.43 ± 3.98 a
Isobutanol	111.91 ± 13.75 a	108.67 ± 8.38 a	117.60 ± 16.90 a	91.12 ± 9.01 a	119.64 ± 19.14 a	108.97 ± 11.07 a	103.82 ± 8.14 a	95.79 ± 0.24 a	102.27 ± 0.76 a	107.86 ± 9.50 a
2-Phenylethanol	95.71 ± 11.50 b	97.67 ± 2.52 b	102.26 ± 8.25 ab	93.00 ± 9.61 b	133.44 ± 11.26 a	110.96 ± 15.75 ab	104.37 ± 3.19 ab	97.21 ± 11.04 b	94.35 ± 4.98 b	98.23 ± 3.70 b
Esters										
Ethyl acetate	110.76 ± 14.60 a	111.98 ± 13.10 a	93.62 ± 12.47 a	105.66 ± 16.24 a	99.29 ± 16.89 a	121.22 ± 16.41 a	94.10 ± 15.85 a	99.37 ± 16.37 a	101.89 ± 6.47 a	95.27 ± 9.33 a
Isoamyl acetate	5.25 ± 0.22 ab	5.20 ± 0.04 ab	5.02 ± 0.08 b	6.27 ± 0.43 a	6.06 ± 0.27 ab	5.50 ± 0.49 ab	5.85 ± 0.52 ab	5.11 ± 0.16 b	5.31 ± 0.22 ab	4.98 ± 0.08 b
Ethyl hexanoate	1.18 ± 0.07 bc	1.2 ± 0.00 bc	1.15 ± 0.05 c	1.47 ± 0.05 a	1.35 ± 0.06 ab	1.28 ± 0.05 abc	1.33 ± 0.07 abc	1.15 ± 0.05 c	1.22 ± 0.06 bc	1.15 ± 0.02 c
Ethyl lactate	2.54 ± 0.10 b	2.56 ± 0.08 b	3.24 ± 0.08 ab	2.62 ± 0.14 ab	3.05 ± 0.31 ab	3.27 ± 0.43 a	2.91 ± 0.07 ab	3.27 ± 0.21 a	3.07 ± 0.02 ab	2.71 ± 0.06 ab
Ethyl octanoate	0.37 ± 0.00 bcd	0.32 ± 0.01 cd	0.28 ± 0.05 d	0.59 ± 0.03 a	0.47 ± 0.04 ab	0.39 ± 0.03 bcd	0.42 ± 0.06 bc	0.32 ± 0.05 cd	0.33 ± 0.04 bcd	0.3 ± 0.03 cd
Diethyl succinate	0.30 ± 0.06 abc	0.36 ± 0.02 abc	0.31 ± 0.02 abc	0.40 ± 0.05 ab	0.42 ± 0.03 a	0.44 ± 0.06 a	0.40 ± 0.02 ab	0.20 ± 0.03 c	0.29 ± 0.08 abc	0.25 ± 0.00 bc
2-Phenylacetate	1.32 ± 0.21 bc	1.05 ± 0.01 c	1.11 ± 0.10 c	2.05 ± 0.25 ab	2.44 ± 0.35 a	1.30 ± 0.05 bc	2.08 ± 0.20 ab	0.90 ± 0.15 c	1.14 ± 0.24 c	1.12 ± 0.15 c

Table A2.4. Continued.

	Yeast derived			Yeast derived with mineral salt				Mineral salt	Controls	
	Y – 1	**Y – 2**	**Y – 3**	**YM – 1**	**YM – 2**	**YM – 3**	**YM – 4**	**M – 1**	**C+**	**C-**
Volatile acids										
Propionic acid	2.17 ± 0.20 a	2.55 ± 0.02 a	2.35 ± 0.16 a	2.79 ± 0.14 a	2.63 ± 0.05 a	2.71 ± 0.26 a	2.53 ± 0.15 a	2.36 ± 0.25 a	2.66 ± 0.26 a	2.32 ± 0.15 a
Isobutyric acid	0.91 ± 0.15 ab	1.09 ± 0.02 a	1.03 ± 0.13 ab	0.78 ± 0.04 b	0.90 ± 0.13 ab	0.89 ± 0.01 ab	0.91 ± 0.06 ab	0.78 ± 0.03 b	0.89 ± 0.02 ab	0.92 ± 0.03 ab
Butyric acid	1.06 ± 0.04 abc	1.20 ± 0.05 ab	1.10 ± 0.05 abc	1.21 ± 0.04 a	1.20 ± 0.07 a	1.18 ± 0.07 abc	1.16 ± 0.01 abc	1.03 ± 0.03 c	1.10 ± 0.03 abc	1.04 ± 0.01 bc
Isovaleric acid	0.80 ± 0.14 a	0.90 ± 0.06 a	0.75 ± 0.09 a	0.69 ± 0.05 a	0.77 ± 0.10 a	0.73 ± 0.02 a	0.75 ± 0.06 a	0.65 ± 0.04 a	0.73 ± 0.01 a	0.77 ± 0.03 a
Valeric acid	0.29 ± 0.01 ab	0.33 ± 0.02 a	0.32 ± 0.01 a	0.30 ± 0.01 ab	0.25 ± 0.01 b	0.31 ± 0.01 a	0.29 ± 0.01 ab	0.31 ± 0.03 a	0.34 ± 0.01 a	0.31 ± 0.00 a
Hexanoic acid	1.63 ± 0.07 de	1.71 ± 0.01 cde	1.5 ± 0.14 e	2.44 ± 0.08 a	2.14 ± 0.11 ab	1.96 ± 0.05 bcd	2.06 ± 0.18 abc	1.62 ± 0.08 de	1.73 ± 0.19 bcde	1.58 ± 0.13 de
Octanoic acid	1.04 ± 0.10 cd	1.09 ± 0.03 bcd	0.92 ± 0.11 d	1.71 ± 0.06 a	1.40 ± 0.09 abc	1.27 ± 0.07 bcd	1.42 ± 0.18 ab	1.08 ± 0.08 bcd	1.16 ± 0.14 bcd	1.07 ± 0.10 bcd
Decanoic acid	0.88 ± 0.02 bc	0.84 ± 0.03 bc	0.79 ± 0.05 c	1.07 ± 0.01 a	0.98 ± 0.05 ab	0.95 ± 0.07 abc	0.98 ± 0.06 ab	0.85 ± 0.07 bc	0.88 ± 0.05 abc	0.86 ± 0.04 bc
Acetic acid	229.59 ± 31.41 ab	276.12 ± 14.44 a	238.90 ± 18.83 ab	215.60 ± 10.44 ab	185.96 ± 9.81 b	238.68 ± 26.66 ab	241.11 ± 7.78 ab	248.45 ± 2.39 ab	263.79 ± 21.02 a	279.02 ± 20.36 a

Chapter 3

General discussion and conclusions

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3.1 General discussion

The use of selected non-*Saccharomyces* yeasts for wine fermentations has been shown to positively impact wine quality (Jolly *et al.* 2014; Padilla *et al.* 2016). However, these yeast species often compete for nutrients with *Saccharomyces cerevisiae*, occasionally resulting in slow or stuck fermentations and suboptimal production of aroma compounds (Medina *et al.* 2012; Gobert *et al.* 2017). This study aimed to investigate different commercial yeast nutrients and how they could benefit selected non-*Saccharomyces* wine yeasts in single and mixed (sequential) fermentations. As far as could be determined, this was the first study that investigated nitrogen supplementation at the same level but with different content for these yeasts in single and mixed fermentations.

From the nitrogen content of the yeast nutrients it is clear that they were formulated for *S. cerevisiae*. The nutrients that had high amino acid concentrations were abundant in amino acids preferred by *S. cerevisiae* (Ljungdahl and Daignan-Fornier 2012). The results in synthetic must showed that the nutrients had a greater effect on fermentation kinetics than on the growth of the yeasts for single fermentations. Nutrient treatment Y2 was shown to improve the fermentation kinetics, both single and mixed fermentations, for all the yeast species investigated. There appeared to be a correlation between the final non-*Saccharomyces* and *S. cerevisiae* cell count for some of the mixed fermentations, with *Torulaspora delbrueckii* having a negative correlation and *Pichia kluyveri* a positive correlation. Although there was no discernible pattern observed for *Metschnikowia pulcherrima* sequential fermentation, it appeared that higher final *M. pulcherrima* cell counts correlated with slower fermentation. This study also established that synthetic must is not a true representative for grape must. When *M. pulcherrima* sequential fermentations were repeated in Chenin blanc must, they were faster and different nutrients better supported the growth of M. pulcherimma. *M. pulcherrima* also reached greater cell counts within the first 48 h, double than observed in synthetic fermentations, however, these cell counts declined fast after *S. cerevisiae* was inoculated. The

results also showed that nutrient selection can improve the concentration of desirable esters in wine. This study found that nutrient selection can greatly influence the implantation of different non-*Saccharomyces* yeasts and potentially improve the sensory properties of wine.

The nutrients in this study were primarily investigated for their YAN contributions, specifically the free ammonium, and amino acids that could be quantified. It has however been reported that yeast derivatives, found in most of the nutrients, also contain lipids, mannoproteins, bound amino acids and other micronutrients (Belviso *et al.* 2005; González-Marco *et al.* 2010; Del Barrio-Galán *et al.* 2012; Pérez-Magariño *et al.* 2015; Kevvai *et al.* 2016). Beside the additional nitrogen available from bound amino acids that the yeast could utilise, lipids can also improve fermentation kinetics and growth of yeasts (Munoz and Ingledew 1989; Belviso *et al.* 2005; Kevvai *et al.* 2016). Furthermore, a recent study found that micronutrient limitations can influence fermentation of *S. cerevisiae* in a nitrogen-dependant way (Duc *et al.* 2017). Therefore, it is possible that the results observed was not exclusively due to the quantified YAN and could also be a result of a more complex interaction and utilisation of other nutrients by the yeast species.

This study, as an exploratory investigation of the impact of complex yeast nutrients on selected non-*Saccharomyces* yeasts, has other limitations than already discussed. These include limited sampling points and metabolite analysis. Due to the limited sampling points during the fermentation the complete evolution of yeasts could not be observed. It is not clear exactly how long the non-*Saccharomyces* yeasts were able to persist in the mixed fermentations or whether the cell death observed on day eight were gradual or sudden. The YAN utilisation by non-*Saccharomyces* yeast prior to *S. cerevisiae* inoculation were also not investigated and could improve current understanding of the nitrogen uptake by these yeasts, and also provide insight into the competition for nutrients. This study also only investigated the volatile profile at the end of fermentation for *M. pulcherrima* sequential fermentation. Besides investigating the volatile profile of the other non-*Saccharomyces* yeast mixed fermentations, the single yeast fermentations should also be investigated to improve the understanding of which volatiles each

yeast produce as a result of nutrient supplementation, and to enlighten the contribution of each yeast in the mixed fermentation to the volatile profile. Most of this research was conducted in synthetic must and this was shown to not be an appropriate matrix with regards to investigation of nutrient supplementation, therefore the other non-*Saccharomyces* yeast fermentations should also be repeated in real grape must. In the current study, only one strain of each yeast species was investigated and might not be a true representative for the entire species.

3.2 Future work

Future studies are required to strengthen and expand the results. The lipid, mannoprotein, bound amino acids and other micronutrient content of complex yeast nutrients should be quantified in further studies. This could improve the current insight into the results observed in this study. Future studies should also investigate higher initial YAN concentrations, different nutrient concentrations and possibly timing of nutrient addition. These factors have been found to have an impact on both yeast growth and fermentation kinetics as well as the production for volatile compounds for *S. cerevisiae* with ammonium supplementation (Torrea *et al.* 2011; Vilanova *et al.* 2012; Seguinot *et al.* 2018). Due to variation in grape must, it is also important that other cultivars, white and red, should be investigated (Moreira *et al.* 2011). As most red cultivars also undergo malolactic fermentation, and with recent interest of non-*Saccharomyces* yeast interaction with *S. cerevisiae* and lactic acid bacteria (Du Plessis *et al.* 2017; Du Plessis *et al.* 2019), nutrients should also be considered for investigation in these studies.

Complex yeast nutrients should also be investigated for different strains of non-*Saccharomyces*, as well as *S. cerevisiae*, to highlight whether strain variation exist for nutrient supplemented fermentations. The volatile profile of different nutrient treatments for single yeast fermentation should also be investigated. This data can be used to evaluate whether the changes observed in volatile concentrations in sequential fermentations are the result of yeast-nutrient or yeast-yeast interaction, or a more complex interaction. The volatile profile of the other non-*Saccharomyces* yeasts in this study should also be investigated.

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